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Probing electromechanical coupling in collagen at the nanoscale via scanning probe microscopy

by

Denise Denning

The thesis is submitted to University College Dublin in fulfilment of the requirements for the degree of Doctor of Philosophy

School of Physics

Head of School: Prof. Padraig Dunne
Principal Supervisor: Dr. Brian Rodriguez

September, 2014
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Abstract

Electromechanical coupling is ubiquitous in nature and is a functional characteristic in a large range of inorganic and organic materials, including collagen type I – a fibrous protein abundant in mammals. Understanding the biofunctionality of electromechanical coupling in its linear form – piezoelectricity, has been a topic of research spanning over seven decades and yet many questions still remain unanswered. Piezoelectricity in bone and connective tissues such as tendon has been investigated at the macroscopic scale since the discovery of piezoelectricity in bone in 1957 and induced currents via the piezoelectric effect have been shown to activate the healing process in tissues under tension. Biological systems consist of complex hierarchical structures which results from a high degree of organization from the macroscale down to the nanoscale. These complex structures, however, make quantitative piezoelectric measurements difficult. Therefore, there exists a need to understand these processes at the individual protein level – i.e. at the nanoscale. In this thesis, a voltage-modulated form of atomic force microscopy called piezoresponse force microscopy is utilized to investigate the counterpart which is responsible for piezoelectricity in bone and connective tissues – collagen. The polar properties of collagen were revealed at the nanoscale and were shown to result in a highly complex polar architecture in natural tissue, which is important for understanding tissue development. Shear piezoelectricity was discovered to persist in engineered collagen hydrogels, a study intended to highlight the importance of replicating both structural and functional properties in replacement tissues. The electromechanical properties of collagen
type II were investigated which were previously unknown. Collagen type II was shown to be a shear piezoelectric, exhibiting an angle dependence of the piezoelectric signal with cantilever-fibril angle. In addition, the piezoelectric tensor of collagen type I was determined at the nanoscale. Most piezoelectric coefficients measured were higher than those previously reported at the macroscopic scale. The new local tensor here will be useful for future studies which are concerned with the biofunctional implications of piezoelectrically-induced charges in collagen at the nanoscale.
Declaration

I hereby certify that the submitted work is my own work, was completed while registered as a candidate for the degree of Doctor of Philosophy, and I have not obtained a degree elsewhere on the basis of the research presented in this submitted work.

________________________
(Denise Denning)
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### Abbreviations

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<td>AFAM</td>
<td>Atomic force acoustic microscopy</td>
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<td>AFM</td>
<td>Atomic force microscopy</td>
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<tr>
<td>BE-PFM</td>
<td>Band excitation piezoresponse force microscopy</td>
</tr>
<tr>
<td>C</td>
<td>COOH or C terminal</td>
</tr>
<tr>
<td>EFM</td>
<td>Electrostatic force microscopy</td>
</tr>
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<td>EM</td>
<td>Electron microscopy</td>
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<tr>
<td>FFT</td>
<td>Fast Fourier transform</td>
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<tr>
<td>FWHM</td>
<td>Full width half maximum</td>
</tr>
<tr>
<td>HOPG</td>
<td>Highly ordered pyrolytic graphite</td>
</tr>
<tr>
<td>KPFM</td>
<td>Kelvin probe force microscopy</td>
</tr>
<tr>
<td>LN</td>
<td>Lithium niobate</td>
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<tr>
<td>LFM</td>
<td>Lateral force microscopy</td>
</tr>
<tr>
<td>LPFM</td>
<td>Lateral piezoresponse force microscopy</td>
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<td>N</td>
<td>NH$_2$ or N terminal</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>Abbreviation</td>
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<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
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<tr>
<td>PFM</td>
<td>Piezoresponse force microscopy</td>
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<tr>
<td>PI</td>
<td>Isoelectric point</td>
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<tr>
<td>RH</td>
<td>Relative humidity</td>
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<tr>
<td>SBF</td>
<td>Simulated body fluid</td>
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<td>SEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>SGP</td>
<td>Strain generated potential</td>
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<tr>
<td>SPM</td>
<td>Scanning probe microscopy</td>
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<tr>
<td>STM</td>
<td>Scanning tunneling microscopy</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>VDF-TrFE</td>
<td>Vinylidene-fluoride trifluoroethylene</td>
</tr>
<tr>
<td>VPFM</td>
<td>Vertical piezoresponse force microscopy</td>
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Chapter 1

Introduction

1.1 Collagen: the most abundant protein in the body

Collagen is an abundant protein in all animals, constituting one third of the overall protein content in humans.\(^1\) It is the main structural protein of the body, present in all extracellular tissues and has extraordinary mechanical properties.\(^2\) The most common structure which collagen and collagenous tissues form is a fibril – a rope-like fiber with a diameter of typically a few hundred nm and a length of up to hundreds of microns. Different fibril sizes, orientations, types, structures as well as the integration of collagen with organic and inorganic constituents all lead to the formation of a variety of tissues with specific functions, such as tough bone, elastic tendon, transparent cornea, opaque...
sclera, soft cartilage, tough dentin, elastic skin, flexible ligaments, elastic blood vessels etc.\[^3\]

Since scientists G. M. Ramachandran and G. Kartha first proposed the triple-helical model for the structure of the collagen molecule in 1954,\[^4\] thousands of papers have been published to date using various techniques such as electron microscopy (EM) and X-ray diffraction (XRD) to investigate the structure of collagen from the tissue down to the molecular level.\[^5\] While the ultrastructure of collagen is now relatively well-understood, certain processes relating to the function of collagen, such as its electromechanical properties, are not so well-understood at these micro/nano length scales. It has been proposed that the electromechanical properties of collagen have biofunctional implications, so understanding these properties at these relevant length scales are of high importance.

1.1.1 The crystalline structure of collagen

Collagen exists in many different forms, fibrillar and non-fibrillar, but one universal component is the presence of the collagen molecule, which appears in all collagenous structures.\[^6\] The most abundant collagen type is certainly type I, found throughout the body except in cartilaginous tissues. The collagen molecule is made up of three left-handed polypeptide strands which are wound into a right-handed triple helix,\[^7\] a cross section of which can be seen in Fig. 1.1. Each polypeptide chain (or \(\alpha\) chain) comprises the repeating amino acid sequence Gly – X – Y, where Gly is glycine, the smallest amino acid, which is always located within the core of the protein. X and Y are typically proline and hydroxyproline, respectively. Hydrogen bonds stabilize the triple-helix structure.\[^2\]
The molecule can be heterotypic, comprising up to three genetically distinct α chains, or homotrimeric, comprising three identical α chains. Collagen type I is a heterotrimer, having two identical α1 chains and a third distinct α2 chain (denoted [α1(I)]2α2(I)), as shown in Fig. 1.1.

![Collagen molecule diagram](image)

**Figure 1.1** Cross section (or top view) of the collagen molecule showing its three polypeptide (α) chains and the relative position of amino acids (G – Glycine). Adapted from [8].

Each collagen molecule has polar bonds, characterized by an amine (NH₂ or N terminal) and carboxyl (COOH or C terminal) terminus at either end. This results in a dipole moment (or polarization) along the long axis of the molecule which is directed from the N to C termini. Most collagen molecules further assemble into fibrillar structures, whereby each molecule assembles laterally via hydrophobic and electrostatic interactions. This leads to each molecule being displaced longitudinally from one another by ~ 67 nm and the resultant fibril having a repeated gap and overlap structure of
the D-periodic distance (67 nm) as shown in Fig. 1.2 (a). The collagen molecule is typically 300 nm in length and 1.5 nm in diameter. An illustration of a cross section of a type I fibril is shown in Fig. 1.2 (b) where each collagen molecule is arranged in a quasi-hexagonal structure, as verified by X-ray diffraction. The hexagonal structure of collagen molecules leads to a lack of inversion center, or being non-centrosymmetric, and having a symmetry class of $C_6$, or point group $\infty$. When the fibril is fully formed, the N to C polarity of the fibril is retained and remains in the direction along the long axis of the fibril.

![Figure 1.2](image)

**Figure 1.2** The molecular structure within a collagen fibril. (a) Arrangement of collagen molecules along the fibril length and (b) cross section of a fibril showing hexagonal assembly of collagen molecules. Adapted from [11].

Covalent bonds (more specifically intermolecular cross-links formed by the enzyme lysyl oxidase) stabilize the laterally adjacent molecules, which are illustrated by the yellow links in Fig. 1.2 (a). These bonds stabilize the arrangement of molecules within the fibril but they also allow for mechanical forces to be transmitted between neighbouring molecules under an applied stress. This is very important for the function of
tissues such as tendon and bone. In the absence of these cross-links, collagen fibrils lose their mechanical strength. A study of young bone showed that a decrease of 10% in the concentration of immature collagen cross-links led to a reduction of 15% in the mechanical strength of bone. Collagen type I fibrils are typically hundreds of nm in diameter and can reach lengths of hundreds of microns. Fig. 1.3 (a) shows an AFM topography image of a collagen type I fibril from bovine Achilles tendon. The D-periodic spacing is visible along on the length of the fibril, which is highlighted by the line profile (solid grey line) in Fig. 1.3 (b). A polynomial fit was used to subtract the background and a sine fit was implemented to calculate a D-periodic spacing (or inverse frequency) of 65.8 ± 0.2 nm. The low symmetry of collagen gives rise to this D-periodic spacing which yields the functional property called piezoelectricity.
Figure 1.3 Topography structure of collagen type I fibril. (a) AFM height image showing typical collagen type I fibril exhibiting D-periodicity. Scale bar in (a) is 200 nm. (b) Solid grey line is a line profile extracted from red line in (a) and solid black line is a sine function fit, used to calculate the D-periodicity.

1.2 Piezoelectricity

1.2.1 Piezoelectricity: a third rank tensor

Piezoelectricity is a property of some non-centrosymmetric materials whereby they generate a charge under an applied load, or conversely, deform under an applied electric field. This phenomenon was first discovered by the Curie brothers in 1880 in Rochelle salt and has since generated an enormous amount of interest in science and technology.
given its ability to perform in a huge variety of applications.$^{18}$ Some applications include transducers,$^{19}$ sensors,$^{20}$ piezoelectric motors,$^{21}$ actuators,$^{22}$ energy harvesters$^{23}$ and piezoelectric surgery$^{24}$ to name a few.

Piezoelectricity in a material arises due to a lack of an inversion center in a materials crystal structure, as illustrated graphically in Fig 1.4. A group of atoms arranged in a hexagonal configuration are shown in Fig 1.4 (a). When a force is applied (as illustrated in Fig. 1.4 (b)) which displaces the atoms relative to each other, an electric polarization is produced. Conversely, if an external electric field is applied to the piezoelectric material, a volume deformation is induced, as shown in Fig. 1.4 (c).

![Figure 1.4 Schematic of the direct and converse piezoelectric effect. (a) Atomic configuration in typical hexagonal material. (b) The material generates charge (electric polarization) under an applied stress (illustrated by arrows) via the direct piezoelectric](image-url)

(a)

(b)

(c)
effect. (c) The material deforms (strains) under an applied field via the converse piezoelectric effect.

Piezoelectricity is the linear coupling between mechanical and electrical energy. The direct piezoelectric effect relates the induced polarization of the material $P$ to the applied stress $X$ via

$$P_i = d^0_{ijk}X_{jk}$$

where $d^0_{ijk}$ is a third rank tensor which describes the piezoelectric properties of the material.\textsuperscript{17} From simple thermodynamic arguments, the converse process has an equivalent piezoelectric tensor.\textsuperscript{25} The converse piezoelectric effect relates the induced strain $S$ of the material to the applied electric field $E$ via

$$S_{jk} = d^0_{ijk}E_i$$

Of the 32 crystal classes, 21 are non-centrosymmetric and 20 are piezoelectric.\textsuperscript{17} 10 crystal classes in this group are also pyroelectric, having an electric polarization when heated or cooled. Of the pyroelectric group there also exist some crystals which exhibit ferroelectricity, the ability to reverse the electric polarization with an external electric field. This phenomenon has recently been reported in a range of biological materials, including the aortic wall,\textsuperscript{26} nacre seashells\textsuperscript{27} and crystalline $\gamma$-glycine\textsuperscript{28} – the smallest amino acid.

1.2.2 Origin of piezoelectricity in collagen

The cross-sectional arrangement of collagen molecules within a fibril has a hexagonal structure,\textsuperscript{11} as discussed in section 1.1.1, which leads to collagen having piezoelectric...
properties described by a class 6 piezoelectric tensor (as determined macroscopically\textsuperscript{29}) via

\[
d_{ijk}^0 = \begin{pmatrix}
0 & 0 & 0 & d_{14}^0 & d_{15}^0 & 0 \\
0 & 0 & 0 & d_{15}^0 & -d_{14}^0 & 0 \\
d_{31}^0 & d_{31}^0 & d_{33}^0 & 0 & 0 & 0 \\
\end{pmatrix}
\] (3)

Crystal symmetry reduces the number of piezoelectric coefficients which are present in a material. The symmetry considerations for the structure of collagen (hexagonal) lead to 4 piezoelectric coefficients – the longitudinal coefficient $d_{33}^0$, the transverse coefficient $d_{31}^0$, and two shear coefficients $d_{14}^0$ and $d_{15}^0$. The tensorial nature of piezoelectricity leads to the induced electric polarization charge (or conversely the induced deformation) to be highly direction and magnitude dependent. How this affects the piezoelectric measurements will be discussed in chapters 2 and 7 in this thesis.

1.2.3 Wolff’s Law – relevance of piezoelectricity in the body

The functional role of piezoelectricity in collagen is a topic which has been under debate since its discovery in bone in 1957.\textsuperscript{30} Research over the years has proved exclusively that bone is an organ which adapts itself relative to its mechanical environment.\textsuperscript{31} Piezoelectricity in bone has been hypothesized to be involved in the ability of bone to adapt under applied loads via Wolff’s Law.\textsuperscript{30, 32-34} Strain generated potentials (SGPs) – the generation of an electric field due to an applied stress, have been observed in both dry and wet bone and are considered to play an important role in bone remodeling, but the origin of this mechanism is not fully understood.\textsuperscript{35} Another phenomenon known to contribute to SGP is the streaming potential, where an electric current is induced when a
force gradient is present. The current originates from electrolytes flowing through the porous structure of bone. The SGP via a streaming potential mechanism is described as

\[ V = \frac{\zeta \varepsilon \Delta P}{\sigma \eta} \quad (4) \]

where \( \zeta \) is the zeta potential (electric potential at interfacial double layer); \( \varepsilon \) is the dielectric permittivity; \( \Delta P \) is the pressure gradient; \( \sigma \) is the solution conductivity and \( \eta \) is the fluid viscosity.\(^{36}\) Investigations into the nature of the SGP amplitude in wet bone revealed a dependence of the signal on ionic strength, zeta potential and fluid viscosity, results which are consistent with a streaming potential mechanism, as seen from eqn (4).\(^{36}\) However, subsequent studies revealed that collagen is actually the main contributor to the change in the zeta potential in bone due to an applied load; and collagen does not generate a potential due to the streaming mechanism.\(^{37}\) This suggests that another mechanism, i.e. piezoelectricity, must be responsible for the change in zeta potential with an applied stress. It is probable that piezoelectrically-induced charges result in surface adsorption of ions which leads to the shift in zeta potential. The relationship between the SGP via a piezoelectricity mechanism is

\[ V = \left( \frac{d_{ijk}^0 L}{\varepsilon} \right) B e^{\left( \frac{-\sigma t}{\varepsilon} \right)} \quad (5) \]

where \( d_{ijk} \) is the third-rank piezoelectric tensor; \( L \) is the sample thickness; \( B \) is the applied load and \( t \) is time.\(^{38}\)

The effect of piezoelectricity on demineralized cortical bone was studied using a biomimetic approach by authors Noris-Suárez et al.\(^{39}\) Demineralized bone samples were deformed for a period of 4 weeks while bathed in simulated body fluid (SBF) with undeformed samples used as a control. It was found that after 4 weeks, the undeformed
samples showed a presence of hydroxyapatite (HAp) deposited but no preferential deposition on either side was observed. However, there was a statistically significant increase in deposition of HAp on the compressed side of the deformed collagen compared to that of the tensed side, as shown in Fig 1.5.

Figure 1.5 SEM micrographs of demineralized cortical bone collagen immersed in SBF. (a) Tension deformed side after 4 weeks. (b) Compression deformed side after 4 weeks. (c) Higher magnification micrograph illustration mineral deposition. Reprinted from [37].

These results are extremely interesting since preferential deposition of HAp is demonstrated even in the absence of bone depositing cells (osteoblasts). Previous studies have shown that stressed bone yields a negative surface potential on the compressed side and a positive surface potential on the tensed side. On this basis, it is highly probably that positive calcium ions (Ca$^{2+}$) are attracted to the negative compressed side, which in turn attracts negative phosphate ions (PO$_4^{3-}$), and thus providing the foundation for bone growth.

Piezoelectricity in bone as a mechanism for bone remodeling is actually ideally suited for a physiologically relevant effect due to its highly oriented collagen fibers evoking orientationally specific piezoelectric responses. Other SGP mechanisms do not
explain the complex response of bone to varying magnitudes or types of bone stresses (shear, axial, bending etc.). However, in order to investigate piezoelectricity in collagen as a possible mechanism for bone remodeling/tissue regeneration, it is imperative to understand it at the length scales at which these processes occur – i.e. at the nanoscale.

1.3 Outline of thesis

The aim of this thesis is the study of the electromechanical properties of collagen at the nanoscale in order to further our knowledge on the prevalence of piezoelectricity in collagenous materials, investigate if there is any polar ordering within natural tissues and to quantify the piezoelectric tensor for collagen at the nanoscale to provide a case for further studies of this remarkable phenomenon. Given the strong case of biofunctional significance related to piezoelectricity in collagen and bone, this thesis should provide a framework for further research on the topic – which is growing in exploration with the advent of scanning probe microscopy (SPM) techniques. Studying electromechanical properties at the nanoscale is possible using a voltage-modulated AFM technique called piezoresponse force microscopy (PFM). PFM will be used on numerous collagenous structures in order to map the nanoscale electromechanical properties in various connective tissues, understand better the role of structure and pH on piezoelectricity in collagen, investigate electromechanical properties of assembled collagen structures, type II collagen and to determine the piezoelectric tensor of collagen.

Chapter 2 introduces the methods of atomic force microscopy and piezoresponse force microscopy, which illustrates the basic principles of both techniques with an emphasis on calibration of the instruments with a view for quantitative measurements.
Demonstrating the PFM technique to map the piezoelectric properties ranging from an individual collagen fibril to several collagenous tissues will be presented in Chapter 3. We see in this chapter that the piezoelectric properties in collagenous tissues are consistently complex, highlighting a hidden polar architecture which is not visible with standard microscopy techniques. These results will be useful for better understanding the role of polarity in the development of tissues – since no previous study has investigated polarity in natural tissues (other polarity techniques require staining/destruction of the tissue hence the polarity cannot be visualized in the tissues natural form). Chapter 4 aims to address the question of the effect both structure and pH have on the piezoelectric properties of collagen membranes. The results in this chapter reveal that the high degree of structural order present in periodic fibrils is a prerequisite in the order of magnitude of the piezoelectric signal. We also see that pH plays a role in the strength of the piezoelectric properties of collagen. Since collagen substrates are widely used in the study of in vitro cell behavior, these results are important as they show the functional properties of collagen can change depending on structure and pH – which may influence cell behavior. The absence of piezoelectric quantification in assembled/engineered collagenous tissues is addressed in Chapter 5, where we investigate PFM on an assembled collagen hydrogel and directly compare the results with rat tail tendon. It is seen that piezoelectricity is indeed retained in the assembled hydrogel which could be important for tissue engineering applications regarding the successful replication of a natural tissue for implantation. Chapter 6 seeks to answer the question of whether other collagen types are piezoelectric. In this chapter, collagen type II is investigated for its piezoelectric properties, and how they compare to the more abundant collagen type I.
Collagen type II fibrils are shown to exhibit piezoelectricity of lower magnitude than collagen type I but display a similar orientational dependence of the piezoelectric signal. Since electric stimulation has been shown to improve cartilage (abundant in collagen type II) repair, there may be a role in the piezoelectrically-induced charges which will be generated from collagen type II. The piezoelectric tensor is determined in Chapter 7, a result which finally bridges the gap between the known tensor at the macroscopic scale and the unknown piezoelectric tensor at the nanoscale. These results will be of high importance for further studies on biofunctional implications of piezoelectricity since the electrical triggers generated \textit{in vivo} which influence bone remodeling occur at micro/nanometer length scales.

### 1.4 References

5. Scientific, T. \textit{Science Citation Index and Journal Citation Report}. \textbf{2007}.


Chapter 2

Method

2.1 Atomic force microscopy

The AFM was invented by Gerd Binnig, Calvin F. Quate and Christoph Gerber in 1986, as a nanoscale characterization tool based on the scanning tunneling microscope (STM). Using a sharp probe attached to the end of a flexible cantilever, the AFM utilizes the force interaction between the probe and the sample surface in order to generate a three-dimensional topographical image. This is in contrast to STM which utilizes the tunneling current induced between the probe and conducting/semiconducting sample, and hence AFM can extend nanoscale sample characterization to insulators and biomaterials. The resolution of AFM is dependent on the sharpness of the probe in addition to experimental settings (imaging mode of use, imaging environment, flatness of sample, noise levels etc.) but typically has lateral resolution of ~ 1-10 nm (limited by sharpness of tip) and vertical resolution of ~0.1 nm. One huge advantage of AFM over other imaging techniques such as the conventional scanning electron microscope (SEM), transmission electron microscope (TEM) and other vacuum based microscopes is the ability of AFM to operate in a liquid environment. This is of enormous consequence when considering the investigation of biological materials in physiologically relevant environments.
Another huge advantage coupled with AFM is its ability to investigate numerous other sample properties in addition to topographical information. To date, AFM is now capable of investigating the frictional, adhesion, electrical, viscoelastic, mechanical and elastic properties of samples, to name a few. Since forces are ubiquitous in nature, AFM can be employed in almost any field in science.

2.1.1 AFM operation and modes

The AFM comprises many vital components which are illustrated in Fig. 2.1. The main operational components of an AFM are the sensor (cantilever/tip), detector (laser/photodiode) and the scanning system (controller and feedback electronics).

![Figure 2.1 Schematic of an AFM showing all operational components.](image)

As the tip of the probe is brought close to the surface, a force interaction is induced which results in the deflection of the cantilever. The relationship of the tip-surface force and the subsequent deflection of the cantilever is described by Hooke’s Law

\[ F = -kx \] (1)
where F is the tip-surface interaction force, k is the spring constant of the cantilever and x is the deflection or displacement of the cantilever from its free position. As can be seen from eqn (1), the spring constant of the cantilever can be used to control the magnitude of the force exerted on the sample, which is important for soft biological materials.

As the tip is lowered towards the surface, both long-range and short-range interaction forces are induced. The energy of these forces between the AFM tip and surface can be described by the Lennard-Jones potential as shown in Fig. 2.2.

![Interaction force vs tip-sample distance](image)

Figure 2.2 Interaction force vs tip-sample distance as described by the Lennard-Jones potential. Adapted from [8].

2.1.1.1 Contact mode AFM

In contact mode AFM, the tip is placed in physical contact with the surface. From Fig. 2.2 it can be seen that in this contact region, repulsive forces are dominant. In practice, the tip is raster scanned over the surface having a constant force, which is equivalent to a
defined setpoint value for the deflection of the cantilever. The cantilever deflection is used as the feedback signal, whereby the z-piezo will adjust the height of the tip in order to keep the cantilever deflection constant. The deflection of the cantilever is monitored via a laser reflected off the back of the cantilever and onto a position-sensitive photodetector, as seen in Fig. 2.1. Due to the tip being in constant contact with the surface, there are high lateral forces associated with this mode which can prove difficult for soft biological samples. Contact mode is predominately used in this thesis.

2.1.1.2 Tapping mode AFM

In the case of tapping mode (or intermittent contact mode) AFM, the imaging mechanism is dynamic. An extra shake piezo is used to vibrate the cantilever at a predefined amplitude (typically a few nm) at or near, its resonant frequency as it raster scans the surface. From Fig. 2.2 it is seen that both attractive and repulsive forces are experienced by the tip in tapping mode. As the tip approaches the surface, the amplitude of the cantilever will decrease due to tip-surface interaction forces dampening the cantilever energy. This allows for the use of the cantilever amplitude as the feedback signal. In addition to height and amplitude images generated during a tapping mode scan, there is also phase information obtained. This phase image is the phase shift between the driving oscillation and the resultant cantilever oscillation when interacting with the surface. Extra information about the elastic properties of a sample can be obtained from this image, since the phase shift is sensitive to energy loss/dissipation of the tip to the sample.
2.2 Piezoresponse force microscopy

The scanning probe microscopy family has revolutionized the study of nano/microscopic properties of materials for a wide range of applications such as semi-conductors, polymers, electronics, ferroelectrics, materials and manufacturing, biology and biomaterials. In this thesis, a voltage-modulated AFM-based technique called PFM is used to investigate piezoelectric properties at the nanoscale. PFM was first developed in 1992 by scientists Günther and Dransfeld from the University of Konstanz when they demonstrated the ability of AFM to locally polarize and subsequently image the electromechanical properties of the ferroelectric polymer vinylidene-fluoride trifluoroethylene (VDF-TrFE).  

2.2.1 Principles of PFM

In PFM, a conductive AFM probe is placed in contact with a piezoelectric surface. An ac field and sometimes a dc field, is applied between the conductive tip and bottom electrode

\[ V_{\text{tip}} = V_{\text{dc}} + V_{\text{ac}} \cos(\omega t) \quad (2) \]

which induces surface deformations via the converse piezoelectric effect (described in more detail in section 1.2.1). \( V_{\text{ac}} \) is the probing ac bias at the chosen modulation frequency, \( \omega \). The subsequent surface vibrations are detected via the tip deflection which is monitored by conventional AFM means, i.e. laser reflected from back of cantilever onto a position-sensitive photodetector. The resulting piezoelectric vibration signal, \( A \), is demodulated into amplitude at the first harmonic, \( A_{1\omega} \) and phase shift (between the
driving ac bias and the resultant ac piezoelectric deformations) \((\varphi)\) via a lock-in amplifier (LIA)

\[
A = A_0 + A_{1\omega}\cos(\omega t + \varphi)
\]  

(3)

The amplitude, \(A_{1\omega}\), is directly related to the local piezoresponse underneath the tip and the phase shift, \(\varphi\), is related to the sign of the local piezoelectric coefficient.

2.2.2 Vertical and lateral PFM

It is possible to measure two components of the piezoresponse - the out-of-plane and in-plane piezoresponse respectively. The out-of-plane piezoresponse (called vertical piezoresponse force microscopy or VPFM) is measured by detecting the flexural vibrations of the cantilever\(^9\) whilst the in-plane piezoresponse (called lateral piezoresponse force microscopy or LPFM) is measured via a twisting of the cantilever via frictional forces resulting from an in-plane polarization component.\(^{10}\) These two modes of PFM are illustrated in Fig. 2.3.
Figure 2.3 Schematic of VLPFM and LPFM. (a) In VPFM, the electric field is parallel to polarization axis leading to out-of-plane cantilever deflection. (b) In LPFM the electric field is perpendicular to polarization axis leading to a twisting or in-plane (shear) cantilever deflection.

The measured out-of-plane or in-plane piezoresponse can be written as

\[ A_{1\omega} = A_{el} + A_{piezo} + A_{nl} \]  

(4)

where \( A_{el} \), \( A_{piezo} \) and \( A_{nl} \) are the electrostatic, piezoelectric and non-local contributions to the measured signal \( A_{1\omega} \). \( A_{nl} \) contributions arise from the non-local capacitance interaction between the cantilever and surface.\(^1\) In an ideal case, the \( A_{el} \) and \( A_{nl} \) terms are minimized (e.g. stiff cantilevers \( k > 1 \) N/m) can be used to minimize electrostatic contribution\(^2\) so that \( A_{piezo} \) is the main contributor to the measured signal – this criteria should be met for quantitative piezoelectric calculations. In the (simple) case of \( A_{piezo} \)
being maximum, and measured on a $c^+$ or $c^-$ piezoelectric domain (as is the case in Fig. 2.3 (a)), the resultant piezoresponse can be given as

$$A_{1\omega} = ad_{33}V_{ac} + Q_{333}V_{ac}^2$$  \hspace{.5cm} (5)

where $a$ is the calibration parameter (deduced from lock-in and deflection sensitivity), $d_{33}$ is the longitudinal piezoelectric coefficient (pm/V), $Q_{333}$ is the electrostriction coefficient and $V_{ac}$ is the driving ac voltage.\textsuperscript{13} For good PFM imaging, there should be good tip-surface electrical contact – such that the driving voltage equals, or almost equals, to the surface potential of the piezoelectric (i.e. tip-surface potential drop).\textsuperscript{14} The presence of a contamination layer on the sample and a worn metal coating on the tip/cantilever are just two examples which can lead to bad tip-surface electrical contact and hence, lead to inaccurate PFM results.

2.2.2.1 Background signal and frequency dependence of piezoresponse

Another very important point to note is that quantitative measurements (pm/V) cannot be obtained via a single voltage point (i.e. from a piezoresponse image) due to offsets inherent in the measurement/instrument which lead to a background signal.\textsuperscript{15} It is necessary to overcome this issue by performing voltage ramping experiments and by recording the amplitude whilst in contact with the surface (henceforth called a point measurement). Determining the slope of this point measurement graph yields a piezoresponse value (pm/V) which eliminates the dependency of the measured signal on the background signal.

Another issue which can obstruct quantification of PFM is the frequency dependence of the piezoelectric signal. Fig. 2.4 shows a graph of the VPFM amplitude recorded on
the piezoelectric and ferroelectric lithium niobate (LN) and the non-piezoelectric glass at two voltages (V=1;V=10) where the frequency of the excitation voltage is swept from 0 – 1.8 MHz. The piezoresponse of LN is shown in Fig. 2.4 (a), where many amplitude peaks can be seen as a function of frequency, illustrating the frequency dependence of the PFM signal. However, it is important to note that if the sample is not properly mounted (i.e. with silver paint/glue etc.) acoustic peaks appear in the PFM amplitude, which may contribute to the signal here.

For both voltage points, a large resonant peak can be observed at ~ 350 kHz. This corresponds to the contact resonance of the PFM signal. As the tip is always in contact with the surface for PFM measurements, there exists a frequency at which the tip-sample amplitude becomes high – this is called the contact resonance. The frequency at which this contact resonance occurs is dependent on the spring constant and free resonance of the cantilever – the sample itself does not need to be piezoelectric. This is highlighted in Fig. 2.4 (b) where the PFM amplitude is measured on the non-piezoelectric glass sample. The recorded amplitude is flat (but non-zero) with the exception of the contact resonance peak, which corresponds to the same contact resonant frequency to that seen for LN – highlighting the dependence of the contact resonance frequency on the cantilever specifications only.
Figure 2.4 VPFM amplitude measured as a function of frequency. (a) PFM amplitude measured on the piezoelectric LN and (b) the non-piezoelectric glass at two voltage states (V=1; V=10).

The amplitude of the contact resonance however, is affected by the properties of the sample. A comparison between the amplitude values at the contact resonance between LN and glass (0.172 V and 0.020 V, respectively) reveal the amplitude of LN is an order
of magnitude higher than that on glass. This is due to the contact resonance enhancing the piezoelectric signal – which is absent in the case of glass.

2.2.3 *Calibration*

Quantitative PFM remains a challenging area in the field since its invention as the contact electromechanics of piezoelectric materials is a complex problem.\textsuperscript{11, 16} This section is concerned with various methods to calibrate both the out-of-plane and in-plane sensitivities.

However, in the case of determining the in-plane sensitivity, lateral gain ratio (lateral/vertical gain ratio) needs to be calculated, since the MFP-3D AFM employs a voltage amplifier to the raw lateral signal (gain of ~0.3). This leads to a smaller lateral signal outputted from the controller than what the actual lateral signal is. The lateral gain ratio was determined via obtaining high resolution thermal data in the range of 0 – 1 MHz for both the vertical and lateral signals. The gain ratio (vertical/lateral) in the frequency regions of which LPFM is generally operated (1 – 40 kHz) is shown in Fig. 2.5, which shows there is a gain factor of between 3 and 6 to be applied in LPFM for the 1 – 40 kHz range.
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Figure 2.5 Lateral gain ratio determined from high resolution thermal data with polynomial fit (n = 4) with 68% prediction band.

Once the lateral gain ratio is determined for the frequency chosen in the experiment, the full calibration of the lateral deflection sensitivity can be conducted. Several methods to do this are provided in section 2.2.3.2.

2.2.3.1 Calibrating the out-of-plane signal (VPFM)

Out-of-plane piezoelectric deformations are typically calibrated via the force-curve method\textsuperscript{17} or using $x$-cut quartz.\textsuperscript{13} In the force-curve method, the deflection sensitivity (values typically $\sim 100$ nm/V for cantilever PPP-EFM (Nanosensors)) is obtained from the constant compliance region of a contact mode force curve on a hard substrate (typically glass). The other widely used method is calibration via the commercially available $x$-cut quartz.\textsuperscript{18} This crystal has a known $d_{11}$ coefficient of 2.3 pm/V,\textsuperscript{19} so the application of 1 V to the $x$-cut surface will result in a deformation of 2.3 pm in the $x$-direction. Generally, the actual $d_{11}$ coefficient of the crystal is first measured using a
different distance-sensitive instrument, such as a double-beam laser interferometer. It can then be measured using PFM, where the lock-in output is scaled to the known deformability of the quartz crystal. For both methods of out-of-plane calibration, it is important to repeat the chosen method for each experiment, since the sensitivity is dependent on the cantilever and laser position.

2.2.3.2 Calibrating the in-plane signal (LPFM)

In the case of calibrating in-plane piezoelectric deformations however, the processes are much less trivial. Though there are several methods widely used in the calibration of lateral force microscopy (LFM), they are difficult to implement in the case of LPFM. Examples include adding a mass to the cantilever, scanning against a wedge, and using a piezoresistive sensor, each of which are difficult to adapt for LPFM measurements as they calculate the force sensitivity, not the lateral deflection sensitivity – which is required. There have been a few attempts at resolving this problem, including scaling to a known shear deformation (e.g. LN), a geometry approach, measuring the lateral signal as a function of scan size, and measuring the lateral signal as a function of surface/cantilever tilt.

Scaling the lateral signal to the known shear deformation of a standard crystal (y-cut LN) has been used in the case of calibrating LPFM. When implementing LPFM on y-cut LN, the in-plane component LPFM should be sensitive to only the $d_{15}^0$ piezoelectric coefficient for the correct orientation. Hence, via conducting a point measurement (described in section 2.2.2.1) and subsequently determining the slope should yield a value for the $d_{15}^0$ piezoelectric coefficient of LN. Similarly to calibrating the out-of-plane
sensitivity via $x$-cut quartz as described in 2.2.3.1, the LPFM piezoresponse is then scaled to the known deformation of the bulk value – in this case the $d_{15}^0$ piezoelectric coefficient of LN is 68 pm/V. The effect of load on the LPFM piezoresponse is also investigated, since both large and small indentation forces have been modelled to produce sufficient effects when conducting PFM measurements. Fig. 2.6 shows point measurements on LN obtained at various loads.

![Figure 2.6 LPFM point measurements obtained on $y$-cut LN for loads of 5 – 200 nN.](image)

Figure 2.6 LPFM point measurements obtained on $y$-cut LN for loads of 5 – 200 nN.

The lateral invOLS value calculated to obtain a deformation equivalent to the actual bulk $d_{15}^0$ piezoelectric coefficient of LN was ~ 2900 nm/V, which is ~ 4 times higher than the invOLS calculated using the geometric method. No statistically significant load dependence was observed on the LPFM signal.

Calibrating the lateral sensitivity by a geometry approach was determined by deriving the relation of the out-of plane (measured via force-curve method) sensitivity.
with the in-plane deflection sensitivity via the dimensions of the cantilever. The deflection sensitivities are related via the following equation

\[ R = \frac{2L}{3h} \]  (6)

where \( R \) is the ratio between the out-of-plane and in-plane sensitivity, \( L \) is the length of the cantilever and \( h \) is the combined height of the tip and thickness of the cantilever. A typical lateral invOLS value (cantilever PPP-EFM (Nanosensors)) obtained via the geometric method is \(~ 700 \text{ nm/V}\). This approach however, works on the basis of the vertical and lateral signal gains being the same which may not be the case as described in section 2.2.3.

Another lateral calibration method applied to LPFM is via measuring the slope of the lateral signal as a function of increasing scan size. When recording the lateral signal for trace and retrace scans, torsional curves are obtained which represent the torsion of the tip induced by a given scan distance. Subtracting the trace lateral signal from the retrace signal and plotting against the scan difference yields a graph which can be used to estimate the lateral invOLS. As can be seen in Fig. 2.7, after a certain scan distance (in this case \(~ 100 \text{ nm}\)) the tip is no longer in contact with the surface and sliding occurs.
Method

Figure 2.7 Lateral torsion signal difference plotted as a function of scan distance.

The lateral invOLS from Fig. 2.7 is calculated via obtaining the slope of the unsaturated regime in the graph, which was determined to be $\sim 33 \text{ nm/V}$ (cantilever PPP-EFM (Nanosensors)).

The final method investigated to calculate the lateral sensitivity is through measuring the lateral output as a function of cantilever/reflective surface angle tilt directly,\textsuperscript{26, 27, 30} which was implemented with a standard goniometer. The torsion angle was calculated via tilting the AFM head through a known distance ($t$) and measuring the resultant change in lateral signal, as seen in Fig. 2.8. The orange wedge in Fig. 2.8 represents a stationary reflective gold plate placed at an angle underneath the AFM head. This gold plate was used in place of a cantilever to reflect the laser onto the photodetector. It was placed at an angle of 11° which is the same angle the cantilever holder positions the cantilever at (for MFP-3D system) during AFM experiments.
Figure 2.8 Schematic of AFM head tilt measurement used to determine the torsion angle as a function of lateral signal.

The distance between the front and back legs (labelled F and B in figure respectively) of the AFM head (\(L\)) are also known, hence the tilt angle (\(\alpha\)) can be determined using

\[
\alpha = \arctan \left( \frac{t}{L} \right) \quad (7)
\]

The lateral signal versus AFM tilt angle is shown in Fig. 2.9 which displays the linear dependence of the lateral signal as a function of tilt angle. The lateral invOLS is determined from the slope of this graph, which was calculated to be \(0.0753 \pm 0.0001 \, ^\circ/V\).

Figure 2.9 AFM head tilt angle as a function of measured lateral signal.
In a standard LPFM experiment however, the distance moved by the tip is required, not the torsion angle. To calculate the distance moved by the tip, simple trigonometry can be employed, as shown in Figure 2.10.

For such small angles, the lateral displacement of the tip apex can be related to the torsion angle as

\[ l = H \times \tan(\alpha) \] (8)

Therefore if the tip height \( H \) is known, the lateral displacement can be determined experimentally. A summary of the lateral calibration methods and typical sensitivities for the conductive cantilever PPP-EFM (Nanosensors) is provided in Table 1.

<table>
<thead>
<tr>
<th>y-cut LN</th>
<th>Geometric</th>
<th>Scan size</th>
<th>Goniometer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical lateral Invols (nm/V)</td>
<td>2900</td>
<td>700</td>
<td>33</td>
</tr>
</tbody>
</table>
2.2.4 Orientational dependence of PFM signal

As described in section 2.2.1, PFM is sensitive to the local polarization underneath the tip. If the spontaneous polarization of the sample is along the same direction as the electric field (i.e. the case illustrated in Fig. 2.3 (a)), then the recorded piezoresponse will equal the piezoelectric coefficient (i.e. \( A_{1\omega} = d_{33} \)). However, if the sample has an arbitrary orientation then the measured piezoresponse may actually comprise a combination of piezoelectric coefficients. This along with the transformation of coordinate systems will be discussed in more detail, and for the case of collagen, in Chapter 8. Here, we take a look at the effective longitudinal piezoelectric coefficient \((d_{zz})\) of barium titanate for an arbitrary orientation of the sample.

Barium titanate is a ferroelectric and piezoelectric material consisting of a tetragonal crystal structure at room temperature. The piezoelectric tensor in the case of tetragonal oxides is

\[
d_{ijk} = \begin{pmatrix}
0 & 0 & 0 & 0 & d_{15}^0 & 0 \\
0 & 0 & 0 & d_{15}^0 & 0 & 0 \\
d_{31}^0 & d_{31}^0 & d_{33}^0 & 0 & 0 & 0 \\
\end{pmatrix} (9)
\]

For an arbitrary rotation of the sample along the longitudinal axis, the effective vertical piezoelectric coefficient measured, \(d_{zz}\), can be determined as

\[
d_{zz} = (d_{31}^0 + d_{15}^0)sin^2\theta cos\theta + d_{33}^0 cos^3\theta \quad (10)
\]

From this equation we can see that the effective longitudinal piezoelectric coefficient for an arbitrary rotation, \(\theta\), can actually consist of multiple contributions from several tensor coefficients. This illustrates the importance of taking the sample symmetry and orientation into consideration before attempting to measure specific piezoelectric
coefficients. Fig. 2.11 (a) is a 3D representation of the effective $d_{zz}$ coefficient (plotted via Maple), which elegantly illustrates the maximum value of the $d_{zz}$ piezoresponse is actually at an angle $\theta$ from the spontaneous polarization (indicated on diagram via a black arrow in Fig. 2.11 (b)). A 2D representation of the effective $d_{zz}$ piezoelectric coefficient is shown in Fig. 2.11 (b). It is observed that at an angle $\theta = 52^\circ$, the effective longitudinal piezoresponse is highest. From eqn (8) we can see this is the case because the longitudinal piezoresponse is now a combination of not only the $d_{33}$ piezoelectric coefficient, but also of both the $d_{31}$ and $d_{15}$ coefficients.

Figure 2.11 (a) 3D and (b) 2D representation of the $d_{zz}$ piezoelectric coefficient for tetragonal barium titanate (units are in pm/V).

These piezoelectric surfaces demonstrate how the orientation of the sample being investigated should be known, as the tensorial nature of piezoelectricity makes analysis of the longitudinal or shear signal very complex.
2.3 PFM in biology

Piezoelectricity is ubiquitous in biology, due to the presence of polar bonds and optical activity in biological systems, as well as their low structure symmetry (in some cases). As described in Section 1.2.3, there could be possible biological significance of piezoelectricity in the body, however these theories remain unresolved. This is largely due to the complex hierarchical structure of biomaterials such as bone spanning seven structural levels, which precludes the understanding of piezoelectricity at the level of organization that the relevant cellular processes happen (micro/nanoscale). This has changed with the advent of piezoresponse force microscopy, which now allows for the probing of these properties at the nanoscale.

2.3.1 Nanoscale PFM measurements on bone

PFM was first utilized in the biology world in 2004, where authors Halperin et al. demonstrated the PFM technique on both dry and wet bone.\textsuperscript{31} Piezoelectricity in bone was investigated for the first time in 1957\textsuperscript{32} where dry bone was discovered to behave as a classic shear piezoelectric material at the macroscale. In this study however, the longitudinal piezoresponse was measured on both dry and wet bone resulting in a significant longitudinal piezoelectric coefficient for both. In fact, the piezoelectric response of dry and wet bone was found to be very similar, as seen in Fig. 2.12. This is in contrast to studies providing results which prove the opposite – that wet bone is actually not piezoelectric at all.\textsuperscript{33} The longitudinal piezoelectric coefficient was measured, taking into account the inhomogeneity of the electric field under the PFM tip, for wet and dry bone with the average coefficient given as 8.17 ± 0.55 pm/V. These values are an order
of magnitude higher than that measured for bone macroscopically, indicating a disparity between macro and nanoscale measurements.

![Image](image.png)

**Figure 2.12** Longitudinal piezoelectric response measured in dry and wet tibia. Reprinted from [31].

Since bone is a composite material comprising a highly complex organic and inorganic architecture, it is beneficial to study the piezoelectric counterpart in isolated form – i.e. an individual collagen fibril.

### 2.3.2 Nanoscale PFM measurements on collagen

Piezoelectricity was first demonstrated in individual collagen fibrils at the nanoscale by Minary-Jolandan and Yu in 2009. Superscript 34: Shear piezoelectricity was confirmed in an individual collagen fibril with the PFM phase displaying a unipolar axial polarization throughout the length of the fibril. Fig. 2.13 shows the LPFM amplitude and phase images of a collagen fibril – the bright region in (a) represents the magnitude of shear piezoresponse and the uniform region in (b) represents the polarization of the fibril, which is uniform.
Figure 2.13 First PFM demonstration on an individual collagen fibril (a) LPFM amplitude showing collagen fibril exhibits shear piezoelectricity and (b) LPFM phase demonstrating a fibril comprises a unipolar axial polarization. Reprinted from [34].

This study showed that a rotated fibril caused a 180° phase shift in the PFM phase image, demonstrating the unipolar nature of individual collagen fibrils. The authors also estimated the electric potential distribution on the fibril surface when subjected to a shear stress of 70 MPa – which is the experimentally measured stress needed to strain bone towards its yielding point. The strain generated potential distribution due to piezoelectricity in collagen for this stress was calculated to be ~ 120 mV, which is certainly of a magnitude which can cause bioelectric processes.

These studies highlight the lack of knowledge currently of electromechanical processes at the nanoscale. Given the strong case of biofunctional significance of piezoelectricity in bone, there is a pressing need to understand this process at the nanoscale.
2.4 References


Chapter 3

Electromechanical imaging from fibrils to tissues – visualizing molecular polar order at the nanoscale

3.1 Introduction

Properties of biological materials depend strongly on structure, cross-links, and gradients.\textsuperscript{1} These materials generally contain hierarchically-structured mineral and protein or polysaccharide phases, which combine to yield a material tougher than either constituent part alone.\textsuperscript{2, 3} In bone, the hardness and fracture strength exhibited on multiple length scales is due to the configuration of nanoscale crystals of a hard, mineral phase,
hydroxyapatite, intertwined with softer collagen fibrils, as seen in Fig. 3.1. The arrangement and structure of the fibrils and the ratio and relative orientation of mineral and protein phases determines the unique mechanical properties of the connective tissue. Thus, the structure and organization of individual collagen fibrils contributes to the mechanical and functional properties of the tissue where it is found, such as the transparent cornea, elastic tendon, stiff bone, and soft cartilage.

Figure 3.1 Hierarchical structure of bone. Reprinted from [9].

Collagens are present in all multicellular organisms and in many tissues. The structure of collagen fibrils and collagen molecules are discussed in more detail in chapter 1 but to reiterate, collagen fibrils maintain the N to C polarity of the molecules at the fibril ends.

In the framework of Wolff’s law, as described in section 1.2.3, bone remolds in response to mechanical stress to produce a structure best-suited to withstand the applied
stress. How cells sense mechanical stimuli of different magnitudes, direction, and duration, however, is not fully understood.

Figure 3.2 Theoretical effects of collagen piezoelectricity on bone stiffness under open-circuit conditions. Reprinted from [14].

Since the discovery of piezoelectricity in collagen and the subsequent hypotheses regarding its biofunctional role, a lot of research was geared towards understanding whether a streaming potential mechanism or piezoelectric mechanism was responsible for the strain generated potentials in bone. As described in section 1.2.3, piezoelectricity in collagen is likely responsible for changes in the zeta potential under an applied load, thus indirectly influencing the streaming potential during compression, as graphically illustrated in Fig. 3.2. Thus, understanding the polar orientation of collagen may provide insight into the structure and biofunctional properties of these tissues.
Several techniques, including optical microscopy, EM, and AFM, have been applied to visualize the arrangement and alignment of collagen fibrils. Unlike EM techniques, which require dehydrated fibrils, AFM measurements allow tissues to be imaged in air, liquid, and physiologically relevant environments. Many studies have been undertaken to visualize the structure and to measure the mechanical properties of collagenous tissues and collagen fibrils using AFM, including the assembly of collagen, mechanical and viscoelastic properties and the effect of diseases such as diabetes on collagen ultrastructure. Standard microscopy techniques, however, are not sensitive to the polar orientation of the molecules, the polar direction of a fibril, or the polar architecture of a tissue. Only with EM and immunolabeling has the N to C polar orientation of fibrils been determined in tissues.

The piezoelectric nature of collagen allows the N to C polarity to be mapped using PFM. PFM has been developed to probe piezoelectric and ferroelectric properties of ferroelectric materials and has been extended to piezoelectric semiconductors, organic polymers, and biological samples, including collagen, a variety of connective tissues, polysaccharides, and peptide. Recently, ferroelectricity was observed at the nanoscale in aortic arterial walls and in crystalline $\gamma$-glycine demonstrating the importance of electromechanical coupling in biological systems, the implications of which are currently not understood.

A preliminary step in determining the role of piezoelectric biopolymers is to assess the piezoelectric properties of biological tissues, and in particular of the molecular polar order. The polar ordering is indicative of the sign of the piezoelectric coefficients, and thus may be responsible for any directionally dependent biofunctionality. Here, using
PFM, we distinguish the molecular orientation of individual collagen fibrils and determine the polar architecture of rat tail tendon and porcine eye tissues. This technique could be useful in understanding the role of polarity in materials properties and improve our current understanding of cell signaling and mechanotransduction.

### 3.2 Materials and methods

#### 3.2.1 Collagen fibrils on glass slides preparation

Collagen type I from bovine Achilles tendon (Sigma-Aldrich) was swollen in 0.01 M hydrochloric acid for several hours at 0 °C. The solution was then homogenized for 10 min at 9500 RPM, filtered, diluted, and reconstituted in phosphate buffered saline (PBS) to a concentration of 10 µg/mL. 50 µL of the solution was pipetted onto a glass slide and allowed to incubate at room temperature for 10 minutes prior to rinsing in ultrapure water (18.2 MΩcm⁻¹ resistance, Millipore) to avoid salt crystal formation.

#### 3.2.2 Rat tendon and porcine eye tissue sample preparation

Tendon specimens were harvested using a scalpel and removed with tweezers. The tendon was placed on a gold coated mica disk and allowed to dry in air following rinsing in ultrapure water. The dimensions of the tendon, measured via micrometer, were 440 ± 20 µm in diameter and 4.2 ± 0.02 mm in length. For the tendon cross-section, strands of fascicle were embedded in epoxy, cut transversely and polished in a 0.25 µm diamond slurry. The resulting cross-section diameter was measured to be 400 ± 20 µm. The cornea, sclera, and iris tissues were harvested from a pig and similarly embedded in epoxy, cut transversely, and polished. The dimensions of the cornea cross section was
1.88 ± 0.02 mm in length and 220 ± 20 μm in thickness; sclera dimensions were 1.28 ± 0.02 mm in length and 400 ± 20 μm in thickness and the iris dimensions were 4.33 ± 0.02 mm in length and 380 ± 20 μm in thickness.

3.2.3 Piezoresponse force microscopy measurements

PFM was implemented using an Asylum Research MFP-3D AFM equipped with Stanford Research Systems (SR830) and Zurich Instruments (HF2LI) lock-in amplifiers and a Tektronix (AGF320) function generator. To enhance the piezoelectric signal obtained during measurements, high-voltage PFM was used. An amplifier was constructed using an operational amplifier (PA85, APEX model), which amplified the AC excitation signal with a gain of 10. A high voltage PFM Module (Asylum Research) with a gain factor of 22 was also used for some measurements.

During PFM experiments, the conductive AFM probe (DPE 18, Mikromasch) was in contact with the sample (typical imaging force 90 nN) and an AC bias (~20-40 V_{ac} at 22 kHz) was applied (Fig. 3.3 (a)). If an out-of-plane piezoresponse was present, the tip deflects vertically (vertical arrow) resulting in a subsequent vertical deflection of the laser position on the photodetector. If a shear or in-plane piezoresponse was present, there would be a torsional movement of the cantilever (curved arrow) and a subsequent lateral movement of the laser on the photodetector. More details on the principles of PFM can be found in section 2.2.1.
Figure 3.3 LPFM and VPFM schematic with examples of polar orientational imaging of collagen fibrils. (a) Schematic of LPFM and VPFM operation. (b) 3D surface plot of an AFM topography image of two parallel collagen fibrils with simultaneously captured LPFM phase data overlaid. (c) 3D surface plot of an AFM topography image of a collagen fibril bent around with LPFM phase data overlaid. Reprinted from [41].

For collagen type I fibrils, the application of a bias perpendicular to the fibril axis (the length) results in a shear deformation. This is detectible via the torsion of the cantilever. An AFM topography image of two collagen fibrils parallel to each other is shown in Fig. 3.3 (b). Overlaid on this image is the simultaneously captured LPFM phase image data. The top and bottom fibrils exhibit a 180° phase shift. In PFM, a 180° phase shift between two materials (or domains) signifies they are oscillating out of phase with each other and have opposite polar directions. Henceforth, bright phase (+90°) will be assigned to represent a fibril which has an N to C polar direction pointing towards the right of the
page and dark phase (-90°) denotes the N to C polarity pointing towards the left, which is highlighted with arrows on each phase image.

While quantitative measurements are possible, in this study, the focus is placed on the determination of the polar orientation of the tissues under investigation via PFM phase imaging. Both lateral and vertical PFM have been implemented (LPFM and VPFM, respectively).

Lateral PFM calibration was undertaken based on the geometry of the cantilever.\(^{43}\) This calibration method is described in more detail in section 2.2.3.2. This calibration technique works under the assumption that the gain ratio between the lateral and vertical signal is the same. The frequency was chosen to be in a regime where it is thought that the tip will follow the lateral surface movements.\(^{44}\) Vertical PFM was calibrated using the force curve method, which is described in more detail in section 2.2.3.1, to obtain the out-of-plane sensitivity.

### 3.3 Results and discussion

PFM has been implemented to study the polar architecture of collagenous tissues. Each sample described above has been studied via PFM, revealing the polar orientation of the tissues at the fibrillar level.

#### 3.3.1 PFM demonstration on an isolated collagen type I fibril

To demonstrate the principle of PFM on collagenous tissues, individual collagen fibrils have first been studied. As collagen is a shear piezoelectric, LPFM has been implemented to characterize the in-plane electromechanical properties of a single collagen fibril. A
topography image of a single bent collagen fibril with a LPFM phase image overlay is shown in Fig. 3.3 (c). Due to the N to C polarity of collagen the bend in the fibril should result in a 180° shift in the LPFM phase signal at the bent region. The transition in the phase signal expected on either side of the bend is clearly evident, demonstrating the potential this technique has to map the polar orientation of piezoelectric biomaterials. Without any modifications to the sample under investigation (e.g., staining, bleaching, etc. in EM studies), the polar orientation of the biomaterial can be determined.

3.3.2 Polar orientation imaging of rat tail tendon and transversely cut rat tendon

Tendon comprises closely packed, highly aligned, parallel arrays of collagen fibrils, which connect muscle to bone. Tendon contains a small volume of proteoglycans and elastin and is enclosed by epitenon, a connective sheath. An AFM deflection image of as-prepared rat tail tendon is shown in Fig. 3.4 (a). There is no fibrillar structure visible in this image, so bleaching could be used to reveal the fibrils underneath. The LPFM amplitude image (Fig. 3.4 (b)) of the same area, however, confirms shear piezoelectricity in tendon. The piezoresponse in the image appears to originate from individual fibrils, demonstrating that piezoelectricity in tendon derives from collagen fibril piezoelectricity. The amplitude signal varies throughout the image, which is likely due to the fibrils having different depths resulting in varying magnitude of the amplitude signal. It is also possible that regions with lower signal have, e.g., more epitenon on the surface, which would effectively dampen the lateral signal. In addition to confirming piezoelectricity in tendon, the orientation of the fibrils can be visualized from a surface which has no visible collagen fibrils. The LPFM phase image in Fig. 3.4 (c) reveals the polar ordering of the
fibrils in the tissue. There is a predominant +90° phase in this image, but this is not an accurate representation and has resulted from the imaging conditions. The reasons for which are described in more detail below.

Figure 3.4 PFM images of rat tail tendon surface. (a) AFM topography image of tendon surface, inset shows cantilever orientation. Scan direction is parallel to the tissue long axis. (b) LPFM amplitude image of same area as (a) with corresponding scale bar. (c) LPFM phase image displaying polar orientation of fibrils underneath the surface. (d) AFM topography image of a smaller area of tendon obtained from a different location. (e) LPFM amplitude image and (f) LPFM phase image of this area. Scale bar for (a) – (c) is 2 µm, (e) – (g) is 200 nm. Z-scale bar for (a) is 400 nm and (d) is 130 nm. Reprinted from [41].

Fig. 3.4 (d) displays an AFM height image of a smaller scan area. In this image, fibrillar periodicity remains invisible. From the LPFM amplitude image (Fig. 3.4 (e)) of the same area, however, fibrillar piezoresponse is evident with the “domains” having an average
width of 73 nm ± 25 nm (n = 20), a domain being defined as an individual fibril or groups of fibrils with a uniform polarity. The widths of the “domains” in the image are of the same order to that of a single collagen fibril, indicating that the lateral piezoelectric signal originates from individual fibrils in the tendon. The LPFM phase image (Fig. 3.4 (f)) of this region displays the anti-parallel polar ordering of the fibrils observed in Fig. 3.4 (c) where each phase domain can be attributed to a single collagen fibril. Since this observation is not discernible in the larger size scale PFM phase image, we assume we may need higher resolution/improved imaging conditions to resolve the phase domains of individual fibrils at this scan size. Each neighbouring fibril has an opposite polar orientation, an observation made before in fixed fascia tissue\textsuperscript{23} where groups of fibrils exhibited opposite polar orientations, but not single fibrils, as in this case.

As PFM only probes a small volume underneath the surface of the material (typically ~ 50 nm but dependent on dielectric properties of sample and applied voltage),\textsuperscript{47} additional PFM studies of rat tail tendon cross sections were undertaken to investigate if this observed anti-parallel polar ordering feature is present throughout the thickness of tendon tissue. VPFM is used on this sample as we are investigating the transverse cut of collagen fibrils, which have a non-zero $d_{33}^0$ piezoelectric coefficient,\textsuperscript{48} giving rise to an out-of-plane piezoresponse in this setup. It is important to note also that the maximum signal for the $d_{zz}$ piezoelectric surface (effective out-of-plane piezoresponse) for collagen is at a 45° angle from the fibril axis.\textsuperscript{32} Using the previously calculated macroscopic piezoelectric coefficient values of collagen,\textsuperscript{48} the $d_{zz}$ piezosurface was plotted using Maple, as seen in Fig. 3.5.
Figure 3.5 Orientational dependence of the effective $d_{zz}$ piezoelectric coefficient. (a) 3D and (2D) representation of the effective longitudinal $d_{xx}$ piezoelectric coefficient for collagen (units are in pm/V).

As the orientation of the collagen sample changes along the angle $\theta$, the effective $d_{33}$ piezoelectric signal changes. This is due to the tensorial nature of piezoelectricity and will be discussed in more detail for collagen in chapter 8.

While the values are based on macroscopic measurements, they demonstrate the orientational dependence of the measured piezoelectric signal is non trivial, as seen in section 2.2.4. It is worth noting that while the structure of collagen molecules within a fibril have been shown to have a hexagonal arrangement (see section 1.1.1), it has not yet been proven exclusively that this is solely the origin of piezoelectricity in collagen. Therefore modeling the piezoelectric properties of collagen via a tensor of $C_6$ symmetry
(for hexagonal materials) may not describe the electromechanical behavior of collagen in their entirety.

Figure 3.6 PFM images of a cross section of rat tail tendon. (a) AFM topography image of cross section surface. (b) VPFM amplitude and (c) VPFM phase image of area in (a). (d) AFM topography image of smaller area in (a), inset is AFM deflection image of same area as (d). White arrows highlight areas corresponding to trenches. (e) VPFM amplitude and (f) VPFM phase image of same area as in (d). Scale bar for (a) – (c) is 2 µm, (d) – (f) is 200 nm. Inset in (e) scale bar is 200 nm. Z-scale bar for (a) is 500 nm and (d) 120 nm. Reprinted with [41].

In the tendon cross section, no clear periodic structure is visible in the topography image in Fig. 3.6 (a). This might be expected given collagen fibrils align along the tendon axis with few aligning transversely, thus there is no clear structure associated with cross-sectioned fibril ends at this scan size. The VPFM amplitude image is shown in Fig. 3.6 (b) which confirms the presence of an out-of-plane piezoresponse. Similarly to Fig. 3.4
(b), a varying signal is observed throughout the image, with some bundles or domains having a larger signal. This could be due to fibrils present on the surface having different depths due to sample preparation or dehydration of the sample. A smaller scan size AFM topography image (Fig. 3.6 (d)) from a different area of the tissue again reveals few discernible details on the surface structure; however, in the deflection image (inset), there are small circular trenches (highlighted via white arrows) with an average diameter of 170 ± 56 nm (n = 15), which could correspond to the cross-section of individual or bundles of collagen fibrils. Fig. 3.6 (e) displays a smaller scan size VPFM amplitude image of a region in (Fig. 3.6 (b)). Piezoelectric domains in this image have an average width of 301 ± 131 nm corresponding to a bundle of ~ 2-4 fibrils. As a high out-of-plane signal is observed in this image, it can be assumed that the orientation of the fibrils in this area are between 45° and orthogonal with respect to the page plane. The VPFM phase image (Fig. 3.6 (f)) displays the orientation of the domains visible in Fig. 3.6 (b). The existence of domains here is significant as it demonstrates that the anti-parallel polar ordering which is present on the surface of tendon is translated throughout the thickness of the tissue. While the existence of polar ordering has been demonstrated previously by electron microscopy and second harmonic generation, the spatial distribution of polarity in tissues remains unknown. This includes knowledge on the size distribution of fibrils which would allow for a prediction of polar order in macroscopic tissues using, for example, a Markov-chain mechanism of fibril growth. While little is known about the influence of piezoelectricity in biological systems or whether it plays a role in mechanotransduction, the observation of an anti-parallel polar ordering of collagen fibrils in native tissue may help shed light on these questions.
3.3.2.1 Histogram analysis to determine polarity ratio in tendon

A histogram analysis is used in order to make a quantitative measure of the ratio of $+90^\circ$ and $-90^\circ$ pixels in a PFM phase image, which in turn represent the polarity ratio of domains. Each pixel in the phase images is plotted in histogram form where two peaks, at $+90^\circ$ and $-90^\circ$, are expected. The difference in intensity of these peaks combined with inspection of the AFM images gives an indication of the ratio of fibrils with a $+90^\circ$ polarity to those with a $-90^\circ$ polarity. A histogram is taken of the small scan size PFM phase image (Fig. 3.4 (f)) of rat tail tendon, which is displayed in Fig. 3.6 (a). Here, there is a 9% difference between the $+90^\circ$ and $-90^\circ$ peaks, illustrating that there is an almost equal amount of $+90^\circ$ polarity fibrils to $-90^\circ$ fibrils. The ‘domains’ of fibrils in the image from which this histogram is taken, however, have widths of approximately one individual fibril, demonstrating that an anti-parallel polar ordering phenomenon down to the fibrillar lever exists on the tendon surface.

![Histograms](image)

Figure 3.7 Phase histograms taken from small scale LPFM images from the surface of rat tendon and cross-sectioned rat tail tendon. The histogram in (a) is taken from phase image in Fig. 3.4 (f) representing a ratio of opposite polarizations of fibrils in a rat tendon

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image. The histogram in (b) is taken from Fig. 3.6 (f) representing the ratio of opposing polarities for cross sectional rat tendon. Reprinted from [41].

The same histogram analysis is used for the cross-sectioned rat tail tendon sample to investigate if a similar polar ordering trend persists throughout the thickness of the tissue. Fig. 3.7 (b) displays a histogram of the pixels in Fig. 3.6 (f). 42% more pixels represent fibrils with a polarity pointing out of the page (-90°) than fibrils with a polarity pointing into the page (+90°). While the difference in +90° fibrils and -90° fibrils is larger here than on the surface of tendon, the phase images and histograms demonstrate that there are significant populations of fibril domains with opposite polarity and that these exist throughout the thickness of tendon tissue. More complex collagenous networks will now be investigated, as PFM is used to probe the polar ordering in porcine eye tissues.

3.3.3 Polar orientation imaging of porcine sclera

The cornea and sclera comprise the outer shell of the eye and are structured to withstand internal and external forces. Both primarily contain collagen fibrils, but many EM studies of the cornea and sclera have revealed that the collagen type and the distribution of fibrils are quite different in the two tissues. X-ray microscopy, EM, optical microscopy and AFM have shown that the sclera is composed of collagen fibrils of varying diameters, which organize in randomly oriented lamellae (thicknesses varying from 0.5 – 6 µm) resulting in the opaque characteristic of the sclera.
Figure 3.8 Cornea and sclera investigated by optical and electron microscopy (a) bovine limbus structure displayed by color contrast polarized light microscopy (CCPLM) and (b) cornea structure studied from TEM. Reprinted from [54] and [52].

The difference in fibril orientation between the sclera and cornea is evident in the color contrast polarized light microscopy image of bovine limbus displayed in Fig. 3.8 (a). The limbus is the boundary between the opaque sclera (tissue to the right in Fig 3.8 (a)) and transparent cornea (tissue to the left). The colors in the image illustrate differences in collagen orientation, as can be seen from the randomly oriented lamella in the sclera having a high intensity and the organized lamellae of the cornea having a very low intensity. The high degree of organization present in the cornea can be seen in the TEM image shown in Fig. 2.8 (b). Three lamellae are present here with alternating orthogonal orientation; it is this regular orientation of the lamellae which contributes to the transparency of the cornea.
To date, the polar architecture within eye tissues remains unstudied. Since the polar properties of tissues are involved in the growth of a tissue, mapping the polarity within tissues will be useful to further comprehend the development of tissues. To study the polar orientation of the sclera, both VPFM and LPFM have been implemented.

Figure 3.9 PFM images of porcine sclera. (a) AFM topography image displaying boundary between three lamellae highlighted via dashed white line. (b) LPFM amplitude and (c) LPFM phase image of area in (a). (d) AFM tapping mode amplitude image (zoom in from black box in (a)) with evidence (circled) of D-periodic fibrils on surface. Double white arrows represent fibrillar orientation. (e) VPFM amplitude and (f) VPFM phase image of area in (e). Scale bar for (a) – (c) and (e) – (f) is 1 µm. Scale bar for (d) is 500 nm. Z-scale bar for (a) is 250 nm. Reprinted from [41].

Fig. 3.9 (a) is an AFM height image of an area in the sclera displaying a region containing a boundary between three different lamellae. The dashed white line represents the boundary between the three lamellae and each lamella is numbered. Fig. 3.9 (d) is an
AFM tapping mode amplitude image from the black box highlighted in Fig. 3.9 (a). In this image the lamellae, labeled 2 and 3, exhibit the D-periodicity of type I collagen (circled). The fibrillar orientation is highlighted via double arrows. No D-periodic banding is visible in lamella 1 suggesting an absence of fibrils parallel to the cantilever axis here. Lamellae 2 and 3, for the given fibrillar orientations, would be expected to exhibit a high in-plane signal due to the shear piezoelectricity of collagen. This is confirmed from the LPFM amplitude image in Fig. 3.9 (b), where a high shear piezoresponse is observed in lamella 2 and 3. The fibrillar orientation within these two lamellae is recognizable in this image and follows the orientation of the fibrils highlighted in Fig. 3.9 (d). Piezoelectric domains of varying widths corresponding to fibrils/bundles of fibrils are visible in lamellae 2 and 3 which is consistent with previous studies of the sclera which state there a large variation in fibrillar widths in the sclera. From this image the average domain width is 393 nm ± 253 nm (n = 20). This large standard deviation would suggest that the domain widths consist largely of bundles of fibrils. The fibrillar orientation in lamella 1 is more difficult to visualize from the LPFM amplitude image as it has a lower signal than the other two lamellae and has no recognizable surface features but exhibits a non-zero lateral piezoresponse. By directly comparing the LPFM amplitude image with the VPFM amplitude image (Fig. 3.9 (e)), a more complete visualization of the fibrillar orientation can be seen. A low piezoelectric out-of-plane response is seen in lamella 2, as expected due to the high lateral signal present in Fig. 3.9 (b) and the fibrils having an orientation almost perpendicular to the cantilever axis. However, a high response in the VPFM amplitude for lamella 3 is observed. This is most likely due to cantilever buckling as the orientation of the fibrils in
this lamella is almost parallel to the cantilever axis. The high in-plane response for this lamella can transfer to an out-of-plane cantilever buckling in this orientation which has been studied before. Lamella 1, however, has a larger out-of-plane piezoresponse than the in-plane response seen in Fig. 3.9 (b). This would suggest that the collagen fibrils in this lamella are actually between a 45° and 90° angle to the long axis of the cantilever (as described in section 3.3.2. with tendon), resulting in this high out-of-plane response. The domains in this lamella are 507 nm ± 288 nm (n = 3) in width, indicating the domains represent bundles of fibrils.

By studying the LPFM and VPFM phase images, mapping the polar ordering of the tissue is possible. The N to C polarity of collagen is assigned and labeled in the image. As deduced from the LPFM amplitude image in Fig. 3.9 (b), the lamellae 2 and 3 contain fibrils in parallel plane with the cantilever axis; therefore LPFM phase image (Fig. 3.9 (c)) will be used to describe the polar orientation of these lamellae. Fibrils in the lamella 2 predominantly have a polar direction pointing to the right of the image with a small number of domains of fibrils having a polarity pointing to the left of the page. Lamella 3 has a predominant polar direction which is opposite to lamella 2, again with a small number of fibrils having an opposite polarity. From comparison of the LPFM and VPFM amplitude images as described above, there is evidence that lamella 1 has fibrils which are oriented in the plane orthogonal to the page plane and cantilever axis, therefore the VPFM phase image in Fig. 3.9 (f) will be used to describe its polar ordering. The bright regions in this lamella correspond to fibrils with a +90° polarity (⊗) pointing into the page, and the dark regions correspond to fibrils with a -90° polarity (double circle) pointing out of the page. In comparison to the LPFM phase images from rat tendon, there
appears to be quite different polar architectures between the sclera and tendon. In the sclera, there is no evidence of anti-parallel polar ordering between fibrils; rather it exists only between domains consisting of bundles of fibrils.

3.3.4 Polar orientation imaging of porcine cornea

The cornea consists of highly organized lamellae consisting of aligned collagen fibrils of uniform diameter of 25 nm which contribute to the transparency of the tissue. The lamellae in the cornea all generally align along the tissue surface axis but form orthogonal to each other with the size of lamellae ranging from 0.2 – 3.5 µm.$^{52,56}$

An AFM topography image of a large area of the cornea is displayed in Fig. 3.10 (a). A banding structure is visible (examples outlined via dashed white lines) in this image of different widths which likely corresponds to the well known lamellar structure of the cornea. However, it is difficult to deduce the orientation of the collagen fibrils within these lamellae from the height image. VPFM has been implemented to investigate any differences in the out-of-plane piezoresponse between lamellae which would shed some light on the orientation of collagen fibrils in these lamellae. In the VPFM amplitude image (Fig. 3.10 (b)), there is certainly a visible difference in the out-of-plane piezoresponse between the different lamellae. Lamellae which are visible both in the topography image and in the VPFM amplitude and phase images are highlighted via dashed lines.
Each neighbouring lamella appears to either have a strong and uniform out-of-plane signal or a heterogeneous out-of-plane signal consisting of areas with both weak and strong signals. The polar orientation of the lamella can be investigated through the VPFM phase image (Fig. 3.10 (c)). The lamellar structure visible in both the topography and VPFM amplitude image is still discernible in the phase image. The lamellae which had a strong, uniform piezoresponse in Fig. 3.10 (b) also have a uniform polarity, pointing into the page. The lamellae which showed a heterogeneous piezoresponse consist of regions with opposite polarity. The topography of a smaller scan size area is displayed in Fig.
3.10 (d), showing more clearly the distinct lamellar structure. The lamellae appear to have quite varied widths (1 µm ± 400 nm; n = 15), which previous studies have also reported. Even with a smaller scan size however, the orientation of the collagen fibrils is not easily visualized. In the VPFM amplitude image, again the lamellar structure is evident with every neighbouring lamella having a high out-of-plane piezoresponse. When compared with the VPFM phase image of the same area, a similar trait is observed to that seen in Fig. 3.10 (c). Lamellae which exhibit a high out-of-plane response appear to comprise collagen fibrils with a uniform polar orientation and lamellae with a heterogeneous piezoresponse contains domains of fibrils exhibiting opposite polar orientations. To study this structure more conclusively, both lateral and vertical PFM have been implemented at a boundary between two lamellae.

An AFM topography image showing the boundary between two lamellae in the cornea is displayed in Fig. 3.11 (a). The D-periodicity of collagen is visible in the upper lamella, whereas no periodicity is evident in the bottom lamella. Round raised features are visible in the bottom lamella however, in the AFM deflection image (Fig. 3.11 (d)). Shear piezoelectricity is investigated via the LPFM amplitude image (Fig. 3.11 (b)), which shows there is a significant difference in the in-plane piezoresponse between the two lamellae. The top lamella has a large in-plane piezoresponse signal displaying domains of fibrils with an average width of 267 nm ± 171 nm (n = 20). This indicates the piezoelectric domains consist of bundles of fibrils as the reported collagen fibril width in the cornea is a uniform 25 nm. From the high in-plane signal from the top lamella and the D-periodic fibrils visible in topography, it can be deduced that the orientation of collagen fibrils in this lamella are parallel to the page plane. The bottom lamella, in contrast, has
minimal in-plane piezoresponse. In the corresponding LPFM phase image (Fig. 3.11 (c)), the polar orientation of each piezoelectric domain observed from Fig. 3.11 (b) can be visualized.

Figure 3.11 PFM images of a boundary between two lamellae in porcine cornea. (a) AFM topography image displaying the boundary. (b) LPFM amplitude and (c) LPFM phase image of area in (a). (d) AFM deflection image of same area in (a) revealing D-periodic fibrils in top lamella. (e) VPFM amplitude and (f) VPFM phase image. Scale bar for all images is 500 nm. Z-scale for (a) is 60 nm. Reprinted from [41].

VPFM is used in order to determine the orientation of collagen in the bottom lamella. A high out-of-plane signal would suggest that there are collagen fibrils orthogonal to the top lamella. A low out-of-plane piezoresponse would be expected in the top lamella as shear piezoelectricity has been verified in this lamella from the LPFM image. This assumption is validated in the VPFM image (Fig. 3.11 (e)) as the top lamella gives minimal out-of-plane signal. There is a high out-of-plane signal from the bottom lamella however, which
would suggest the presence of collagen fibrils pointing out of the page where the domains appear to be arranged in bundles of fibrils. However, if we assume the collagen orientation in the bottom lamella is actually in-plane but oriented perpendicular to the top lamella, an out-of-plane piezoresponse would be expected for this cantilever-collagen orientation. In this case, the shear deformation of collagen would give rise to an out-of-plane piezoresponse in the form of cantilever buckling as described in the sclera above. It is important to note, however, that if this orientation was the case, we would expect to see an in-plane piezoresponse from the bottom lamella in Fig. 3.11 (b) as the cantilever would still be sensitive to the induced torsional twisting as seen in Fig. 3.9 (b). While there is a small possibility that this may be the actual collagen configuration, this image reveals the importance of taking all possible cantilever-collagen orientations into consideration. For this case it would be ideal to rotate the sample 90° in order to verify this lamella has only an out-of-plane signal. The structure of the surface of this lamella however does suggest that the fibrils are orthogonal to the top lamella, similar to the case in Fig 2.8 (b). The surface here comprises round raised features, more easily visible in the AFM deflection image (Fig. 3.11 (d)), which are 150 nm ± 28 nm in diameter. While fibrils in the cornea have been shown to have a uniform diameter of 25 nm, it is plausible that these raised features could be the ends of bundles of fibrils, or that they are the correct size but appear larger in diameter due to tip broadening effects. For these reasons, we strongly believe that the bottom lamella is orthogonal to the top lamella. The VPFM phase image in Fig. 3.11 (f) reveals that each neighboring domain present in the bottom lamella exhibits an opposite polar orientation. The orientation of the domains in this
lamella is very similar to that which is seen in the cross section of tendon (Fig. 2.6 (f)), where a layering of the domains is observed.

These images show conclusively that collagen in the sclera and cornea is indeed piezoelectric, that the tissue consists of interwoven and orthogonal lamellae and that the origin of piezoelectricity in the cornea and sclera is due to collagen fibrils. In addition to these observations, the polar orientation of collagen fibrils in the sclera has been visualized. While the presence of a polar direction in collagen fibrils has been studied previously, the organization of the polar orientation of fibrils in native tissues has not been considered in any previous study. No uniform polarity is observed in the sclera or cornea, all lamellae imaged revealed an anti-parallel polar ordering between domains consisting of bundles of collagen fibrils.

3.3.5 Polar orientation imaging of the porcine iris

The iris controls the size of the pupil, and hence the amount of light entering the eye. The stroma contains collagen fibrils interwoven around blood vessels and the iris sphincter. Both VPFM and LPFM have been used to study porcine iris. The iris stroma is the region under investigation in this study as it has collagen in abundance. Fig. 3.12 (a) is a large AFM topography image displaying the surface of the iris stroma. Fibrils are visible and exposed on the surface even with no surface treatment. The D-periodicity of type I collagen can be observed in the image and the fibrils have a uniform orientation.
Figure 3.12 PFM images of porcine iris. (a) AFM topography image of iris surface revealing exposed collagen fibrils. (b) LPFM amplitude and (c) LPFM phase image of area in (a). (d) AFM topography image of smaller area taken from (a). (e) LPFM amplitude and (f) LPFM phase image from area in (e). (g) AFM deflection image of area in (d). (h) 2D FFT taken from area highlighted by white box in (g). (i) Line profile taken from red line in (d) displaying the D-banding of collagen. Scale bar for (a) – (c) is 5 µm, (d) – (g) is 1 µm. Z-scale for (a) is 800 nm and for (d) is 150 nm. Reprinted from [41].

The average fibril width measured from these images is 205 nm ± 65 nm (n = 20). This fibrillar width is much larger than that reported for the cornea and the sclera. Fig. 3.12 (b) displays the LPFM amplitude image, where shear piezoelectricity is confirmed. This is
expected as the collagen fibrils are oriented perpendicular to the long axis of the cantilever, thus the in-plane piezoresponse is maximized. For this reason, no vertical signal was measured, hence only LPFM images are used for investigation of the iris. The in-plane piezoelectric ‘domains’ of fibrils have an average width of 400 nm ± 207 nm (n = 20), indicating that each domain consists of ~1-4 fibrils. The LPFM phase image of this area maps the polar orientation of the iris as shown in Fig. 3.12 (c) where the polar orientation of each domain is visible. Fig. 3.12 (d) is an AFM topography image of smaller scan area within Fig. 3.12 (a) highlighting the individual fibrils. They all show evidence of the 67 nm D-periodicity of type I collagen, demonstrating that the collagen fibrils are completely exposed on the surface. The small scan size LPFM amplitude image (Fig. 3.12 (e)) directly compared with the topography allows us to distinguish that the piezoelectric domains consist of multiple fibrils. While the orientation of the fibrils is visible from the topography image, the LPFM amplitude image also provides information on the orientation. By directly comparing Fig. 3.12 (d) with Fig. 3.12 (e), it can be seen that the piezoelectric domains consist of both a number of fibrils (large white oval) and single fibrils (small white oval). The corresponding LPFM phase image allows for the visualization of the N to C polarity of the fibrils, which in this case organize in domains of opposite polarities. The white circle highlights a region where fibrils are visibly interwoven in between each other. This is a characteristic previously reported in the iris.58

To further confirm D-periodicity in the iris, a 2D FFT analysis of a collagen fibril was undertaken from an area (highlighted by the white box) in the AFM deflection image (Fig. 3.12 (g)) containing a D-periodic collagen fibril. The resulting FFT (Fig. 3.12 (h)) yields two main peaks which correspond to the axial spacing of collagen type I (labeled)
and higher harmonics at a lower intensity. The line profile shown in Fig. 3.12 (i) is taken from the area highlighted by red line in Fig. 3.12 (d). This profile also confirms the presence of the 67 nm D-periodic banding of type I collagen with an average D-period of 66.2 ± 2.3 nm. Given the presence of D-periodic fibrils on the iris surface, which form domains of opposite polarities, iris stroma could be a suitable substrate to investigate the role of collagen polar ordering on cellular interactions in tissues.

Piezoresponse force microscopy has been employed to obtain information on both the polar orientation and the electromechanical properties of collagen fibrils in native rat tail tendon, transversely cut rat tail tendon, and porcine cornea, sclera, and iris tissues. Piezoelectricity has been confirmed in all tissues studied, emphasizing that piezoelectricity is a functional property in all collagenous tissues. By combining vertical and lateral PFM, the polar architecture in tissues can be visualized. Interestingly, no uniform polarity has been observed in any tissue studied. Rat tendon shows evidence of anti-parallel polar ordering down to the fibrillar level, which has been confirmed to persist partially through the thickness of the tissue by studying the cross section of rat tendon. PFM of interwoven and orthogonal lamellae within the cornea and sclera reveals that fibrils form piezoelectric domains consisting of bundles of fibrils exhibiting opposite polar orientations. These results show that collagen fibrils in natural and unmodified tissues form piezoelectric domains with anti-parallel polar orientations. There is no expectation that collagen fibrils will assemble to have either a unipolar orientation or to form piezoelectric domains. While no unipolar orientation was seen in any tissue studied, domains of different size scales were observed in every tissue. It seems plausible to
suggest that this observation is relevant for understanding mechanical and biofunctional
properties of connective tissues.

3.4 References

15. Gale, M.; Pollanen, M. S.; Markiewicz, P.; Goh, M. C. Biophys. J. 1995, 68,
   2124-2128.


58. Muron, A. **2000**.
Chapter 4

Investigation of piezoelectric properties of collagen type I membranes as a function of structure and pH

4.1 Introduction

In the previous chapter, the polar architecture of natural tissues was investigated with PFM. The polar and piezoelectric nature of collagen is described in sections 1.1.1 and 1.2.1. Though the origin of polarity and its resultant piezoelectricity in collagen type I is generally agreed upon in the community, there remains a lack of knowledge regarding the role certain properties (e.g. structure and pH) have on functional (piezoelectric) properties of collagen. Cellular interactions with collagen fibrils in the extracellular
matrix are believed to play a fundamental role in cell proliferation, cell signaling, cell attachment, and tissue restructuring.\textsuperscript{1-4} Yet there is a lot of understanding still required to fully grasp the exact mechanisms for these interactions in the extracellular matrix. Fig. 4.1 displays the results from a study investigating cell behavior on different collagen substrates.\textsuperscript{5} Corneal fibroblasts were seeded on an aligned collagen nanofibril (~30 nm) membrane (called 30A), an aligned collagen membrane with fibrils of a larger diameter (~ 300 nm; called 300A), a collagen membrane with randomly aligned fibrils (called NA) and a glass slide (called Control). The results presented in Fig 4.1 (a) – (d) show that the fibroblasts elongated best on the 30A membrane and in the direction of the fibrillar alignment, and that all fibroblasts elongated better on the collagen substrates than on the control.

![Stained corneal fibroblasts on collagen membranes of varying diameter and alignment.](image)

Figure 4.1 Stained corneal fibroblasts on collagen membranes of varying diameter and alignment. (a) Fibroblasts grown on 30A membrane. (b) Fibroblasts grown on 300A membrane. (c) Fibroblasts grown on a NA membrane and (d) fibroblasts grown on control. Reprinted from [5].

A biochemical analysis in the study also revealed that the expression of several relevant genes of the cornea increased, showing that the collagen membranes can influence matrix synthesis. These results show the influence collagen structure and
alignment can have on cellular behavior, yet more studies should be focused on the exact mechanisms by which collagen interacts with cells.

The need for understanding the structural and functional roles of collagenous materials is of vital importance for tissue engineering applications. Recently, it was shown that glucose suppresses ferroelectricity in aortic elastin, which highlights the importance of understanding the biofunctional role of physical and electrical properties such as piezoelectricity in biomaterials. Previous studies have shown that collagen assembly is greatly affected by pH and also by the presence of electrolytes (e.g., KOH), but the effects of such parameters on the functional properties, including piezoelectricity, of collagen fibrils has yet to be fully investigated.

Figure 4.2 Effect of pH on collagen fibril density on mica. Past the isoelectric point of collagen, no fibrils assembled on the surface. Reprinted from [7].

The effect of pH on the assembly of collagen was investigated previously using AFM. This study observed that collagen fibrils formed until the pH in the environment was
increased towards the isoelectric point (pI) of collagen molecules (pH = 9.1), as shown in Fig. 4.2. At and around this pH the spacing between collagen molecules increased, leading to the authors’ conclusion that collagen molecules repel each other at the pI value of collagen. These results show the importance in the role pH plays in the structure of collagen, yet there is likely an effect on the function of collagen too.

To date, only D-periodic collagen fibrils have been investigated for their piezoelectric properties.\textsuperscript{8-10} One previous study reported piezoelectric response from non-fibrillar collagen,\textsuperscript{11} but no comparison to fibrillar collagen was made and the effect of fibrillar structure on piezoelectricity remains unknown. Since collagen is widely used in neutralized form to investigate cell behavior,\textsuperscript{12} PFM is used to study the electromechanical properties of collagen membranes with different structures comprising aligned fibrils both before and after a neutralization process (critical for cell viability), which alters the pH from acidic to neutral conditions.

4.2 Materials and methods

4.2.1 Preparation of collagen membranes

Two types of collagen samples (Nanoweave, Fibralign) were prepared using a process based on technology developed for liquid crystal display manufacturing\textsuperscript{13-16} in which purified molecular collagen solution in the liquid crystal phase\textsuperscript{17} was sheared onto glass slides. This method provides control over the diameter and alignment of the fibrils. One type of sample comprised aligned collagen fibrils with diameters of several hundred nm in a braided matrix (as seen in Fig 4.1 (b)), which was optically opaque (henceforth
denoted *opaque*). The second type of sample comprised aligned nanofibrils (i.e., having diameters of < 100 nm; and as seen in Fig 4.1 (a)) and was optically transparent (henceforth denoted *transparent*). Differences in fibril diameter and morphology of the collagen membranes were obtained during the preparation by changing the osmolality and concentration of the initial molecular collagen solution. Initial solutions for the *transparent* membrane corresponded to a lower ionic strength than the *opaque* membrane (20 and 80 mOsm/Kg H$_2$O, respectively). Initially, the aligned collagen samples were in acidic form (pH of 2.5), therefore a simple neutralization process was implemented, which includes incubation in Dulbecco’s phosphate buffered saline (pH of 7) for 5 min and rinsing in deionized water to bring the pH of the membrane up to physiological conditions (pH of 7). Directly after the neutralization process, the samples were gently dried with nitrogen and subsequently imaged using AFM and PFM, allowing the effect of structure and pH on the piezoelectric properties to be assessed. By comparing the weight of similar samples before and after heating to 90 °C in vacuum, it was determined that air dried membranes have a water content of between 15-20%.

4.2.2 Topographical and electromechanical characterization methods

Topographical studies of the alignment and structure of the fibrils were performed using a commercial AFM system (MFP-3D, Asylum Research) in both tapping mode and contact mode. During PFM experiments, a 5 kHz AC voltage of 30 V (peak-to-peak) was applied to a conductive cantilever probe (DPE18, Mikromasch), which resulted in local surface deformations via the converse piezoelectric effect. The nominal spring constant and resonant frequency of the cantilevers used was 3.5 Nm$^{-1}$ and 75 kHz, respectively.
Calibration of the vertical sensitivity (via force curve) of the cantilever was performed before and after the experiment (difference of 1%). High voltage PFM imaging was implemented via a custom built high voltage system based on a PA85 amplifier, which allowed for a 10-fold amplification of the excitation AC signal. PFM amplitude images were normalized to the image with the largest piezoresponse for each membrane type (leading to a scaling factor of 10) in order to directly compare the PFM signal between samples before and after the neutralization process.

4.3 Results and discussion

4.3.1 Topographical/structural characterization of collagen membranes

AFM has been used in order to distinguish topographical differences between the transparent and opaque collagen membranes. Fig. 4.3 displays amplitude modulation AFM images obtained for both membranes. An AFM height image of the transparent membrane is shown in Fig. 4.3 (a). The white arrow in Fig. 4.3 (a) represents the direction of fibrillar alignment. The fibrils have small and relatively uniform fibrillar widths (75.9 ± 14.5 nm; n = 20). However, it is possible that the tip radius exceeds the width of the nanofibrils, which would lead to tip broadening. Collagen fibrils assembled in ideal physiological conditions in vitro, or indeed collagen fibrils produced in vivo, give rise to a staggered alignment of collagen molecules resulting in gap zones. This leads to the well-known D-periodicity (~ 67 nm) associated with type I collagen fibrils. AFM height and amplitude images of a 1 × 1 µm² area (Fig. 4.3 (b) and (c), respectively) reveal additional surface details showing that the nanofibrils have a heterogeneous periodic
structure (highlighted by arrows in Fig 4.3 (c). However this periodic structure is not regular and cannot be attributed to the characteristic D-periodic spacing. The AFM height image of the opaque membrane displayed in Fig. 4.3 (d), has a different structure to that seen for the transparent membrane. The fibrillar widths are ~ 5 times larger (391.1 ± 98.7 nm) than those observed in the transparent membrane. There is no apparent periodicity associated with the fibrils of the opaque membrane (from the AFM amplitude image, Fig. 4.3 (e)), indicating they are non-D-periodic type I collagen fibrils. AFM height and amplitude images of a 5 × 5 µm² area (Fig. 4.3 (e) and (f), respectively) confirm the absence of D-periodicity in the opaque membrane.

Figure 4.3 AFM images of transparent and opaque collagen membranes. (a) AFM height image of a transparent collagen membrane (z-scale = 90 nm; scale bar = 500 nm). Inset displays the AFM amplitude image of the same area as shown in (a) (z-scale = 30 nm). (b) AFM height image (z-scale = 40 nm; scale bar = 200 nm). (c) AFM amplitude image, where black arrows highlight non-periodic structure in the fibrils (z-scale = 12 nm). (d) AFM height image of the opaque membrane (z-scale = 550 nm; scale bar = 5 µm). Inset
shows AFM amplitude image of same area as (d) (z-scale = 110 nm). (e) AFM height image of the opaque membrane (z-scale = 400 nm; scale bar = 2 µm). (f) AFM amplitude image (z-scale 50 nm). Reprinted from [18].

In comparison with known tissues, the fibrillar widths of the transparent cornea are 20-50 nm,\textsuperscript{19,20} which is the same order of magnitude as the fibrils in the transparent membrane. In addition, the fibrils in the transparent membrane are oriented, a feature shared by corneal lamellae. Moreover, corneal fibroblasts plated on the transparent membrane have been found to express a normal quiescent phenotype, further highlighting the similarities between the transparent membrane and cornea.\textsuperscript{5} The range of widths in the opaque membrane is in the range found in opaque tendon, which is between 20 - 500 nm.\textsuperscript{21} The uniform width and structure of the fibrils in both membranes highlights to an extent the range of structures that can be made using the liquid crystal approach.

4.3.2 PFM analysis of electromechanical properties of collagen membranes – before and after neutralization

A collagen fibril is a shear piezoelectric.\textsuperscript{8} Therefore, only lateral PFM (LPFM) has been employed for this investigation. AFM and PFM images of the transparent membrane before neutralization are shown in Fig. 4.4. Fig. 4.4 (a) is an AFM height image of a 15 × 15 µm\textsuperscript{2} scan area obtained in contact mode. There is a predominant alignment of the nanofibrils, which is indicated via the white arrow in Fig. 4.4 (a). The piezoelectric properties of the transparent membrane are visualized in Fig 4.4 (b). The nanofibrils are organized into large piezoelectric ‘domains’ whereby a domain is defined as an area of uniform polarization.
Figure 4.4 LPFM images of the transparent collagen membrane before the neutralization process. (a) AFM contact mode height image (z-scale = 170 nm; scale bar = 2 µm). White arrow indicates fibrillar alignment. (b) LPFM amplitude image of area in (a). (c) LPFM phase image revealing polarity of each domain. (d) AFM height image of transparent collagen membrane (z-scale = 70 nm; scale bar = 1 µm). (e) LPFM amplitude image of area in (a). (f) LPFM phase image. White arrows in (d-f) highlight individual fibril showing evidence of polarity reversal due to the change in orientation with respect to the cantilever axis. The yellow arrow in (d) indicates the scanning direction of cantilever. Reprinted from [18].

The averaged piezoresponse value over 10 randomly selected domains was calculated from Fig. 4.4 (b) to be $0.467 \pm 0.105$ a.u. The piezoresponse of each domain was calculated via image processing using $1 \times 1 \mu m^2$ square masks (Igor Pro, Wavemetrics), which allowed the piezoresponse over this predefined area to be averaged. The LPFM images confirm that the shear piezoelectric properties of type I collagen fibrils is
replicated in these transparent membranes. The average size of the domains is 1.65 ± 0.92 µm, indicating there are ~ 20 nanofibrils in each domain. The size of these domains is 1-2 orders of magnitude larger than those seen in either tendon or eye tissues (the domains are sized between 70–400 nm).\(^\text{21}\) The domains take on the structure of the membrane, possibly a result of the induced structural orientation of the nanofibrils, but the domains do not correlate directly with the topography. The LPFM phase image, shown in Fig. 4.4 (c), reveals the polarity of each domain. This is related to the orientation of the dipole in collagen molecules (N to C termini). The 180° phase shift between neighboring domains demonstrates that the direction of the N to C polarity of the two domains is opposite. Analysis of the LPFM amplitude image of a 5 × 5 µm\(^2\) area (Fig. 4.4 (e)) shows there is a ~ 0.25 fold difference between the piezoresponse amplitude of opposing domains. As oppositely oriented domains are expected to exhibit the same amplitude response, this variation could be related to the tip-sample capacitance and to the choice of imaging frequency.\(^\text{22}\) Inspection of the AFM height image and the corresponding PFM phase image reveals that the phase contrast inverts for some fibrils when their orientation changes with respect to the cantilever axis. This polarity reversal of bent collagen fibrils with respect to the cantilever-fibril orientation has been demonstrated previously\(^\text{23}\) and illustrates the orientation dependence of PFM.\(^\text{21}\) An example is highlighted with black and white arrows in Fig. 4.4 (d-f).

After the neutralization process, the pH is adjusted to physiological conditions (pH 7). Fig 4.5 displays the AFM and PFM results for the transparent membrane after neutralization. The topography over a 15 × 15 µm\(^2\) scan area (Fig. 4.5 (a)) is different to that seen before the neutralization process. The fibrils have a similar alignment, however,
a crimped structure also appears, which is generally perpendicular to the direction of fibrillar alignment. The average width of the crimped structure is $1.59 \pm 0.37 \, \mu m$ ($n = 10$), which is one order of magnitude smaller than that seen in natural tendon tissue (10 – 100 $\mu m$). Interestingly, the crimped structures have the same statistical width as the piezoelectric domains ($1.65 \pm 0.92 \, \mu m$). It is possible that there exists a correlation between the size of the crimped structure and the domain size, but verification would require additional investigation. The lateral piezoresponse also changes significantly. The shape and width of the piezoelectric domains (Fig. 4.5 (b)) remain statistically the same, but there is a 1.5 fold increase ($0.703 \pm 0.058$ a.u.; $n = 10$) in the amplitude of the lateral piezoresponse. When averaging only the domains from Fig. 2e, which have a larger response in the before neutralization image ($0.524 \pm 0.034$ a.u.), the increase in piezoresponse compared to the domains from the after neutralization image (Fig. 4.5 (e)) is still statistically significant (1.35 fold). It is possible that this increase in shear piezoelectricity is due to the neutralization process. As the pH of a protein deviates from neutral conditions, the structure of the protein changes. Thus, the functional activity of the protein can drastically decrease. PFM imaging was performed directly after drying the sample.
Figure 4.5 LPFM images of transparent collagen membrane after the neutralization process. (a) AFM height image of a transparent collagen membrane after neutralization (z-scale = 80 nm; scale bar = 2 µm). (b) LPFM amplitude image illustrating a similar domain structure as seen before the process. (c) LPFM phase image showing retention of the polarity of collagen domains. (e) AFM height image (z-scale = 40 nm; scale bar = 1 µm). Dashed white line highlights the crimp structure. (e) LPFM amplitude image of area shown in (d) and corresponding (f) LPFM phase image. Reprinted from [18].

The measurement was repeated on a second transparent membrane, which confirmed the increase (1.2 fold in this instance) in piezoelectric signal after neutralization (data not shown). Hence, based on these measurements, the magnitude of the shear piezoelectric response increases on average 1.35 fold in the transparent membrane after adjustment from acidic to physiological pH. The second measurement was carried out over the course of two hours in order to exclude any changes in the observed piezoelectric signal due to changes in of the moisture content of the sample following the neutralization process. The measured piezoelectric signal did not change during this time. The
calibration of the cantilever was performed before and after each experiment, which revealed there was a maximum of a 1.01 fold increase in signal due to a change in the properties of the tip. The calibration via force curve was also repeated (n = 10) in order to determine the error in calibration, which was 1.03 fold. These measurement errors are all outside the increase in signal, which proves the observed increase was not due to a change in the properties of the tip. The LPFM phase image (Fig. 4.5 (c)) displays the N to C polarity of piezoelectric domains of collagen after adjustment to a physiological pH. An AFM height image of a 5 × 5 µm² area (Fig. 4.5 (d)) highlights the appearance of a crimp structure in the collagen matrix. The crimps are indicated via dashed white lines. The LPFM amplitude image (Fig. 4.5 (e)) of the same area shows the familiar domain pattern and 1.5 fold increase in piezoelectric response compared to that seen before neutralization in Fig. 4.4 (e).

The same measurements were also performed on the opaque collagen membrane. Over a 15 × 15 µm² scan area (Fig. 4.6 (a)), before neutralization, it is again clear that the collagen fibrils in the opaque membrane have a larger fibrillar width to that of the transparent membrane from the AFM height image. Shear piezoelectricity is confirmed in the opaque membrane as seen from the LPFM amplitude image in Fig. 4.6 (b). The non D-periodic fibrils in the opaque membrane also organize themselves into domains where the piezoelectric domains have an average width of 0.390 ± 0.098 µm. The structure of the domains is somewhat similar to the transparent membrane, consisting of domains aligned in the direction of fibrillar orientation. The opaque membrane, however, has a smaller average domain width. The average piezoresponse of 10 domains is 0.605 ±
0.037 a.u., which is larger (before neutralization) than observed for the transparent membrane.

Figure 4.6 LPFM images of non D-periodic opaque collagen membrane before the neutralization process. (a) AFM height image displaying collagen fibrils (scale bar = 2 µm). (b) LPFM amplitude image. (c) LPFM phase image. (d) AFM height image (scale bar = 1 µm). (e) LPFM amplitude image of area shown in (d). Black arrows in (d) and (e) emphasize reduced piezoresponse signal from discontinuities in the collagen matrix. (f) LPFM phase image displaying polarity of domains. The z-scale for (a) and (d) is 700 nm. Reprinted from [18].

The confirmation here that non D-periodic type I collagen fibrils also exhibit shear piezoelectricity is an interesting result given the origin of piezoelectricity in collagen fibrils is attributed to the hexagonal packing of monomers, which may only present in D-periodic fibrils. It has been postulated that the presence of the D-periodicity in type I collagen fibrils is due to the lateral interaction between collagen molecules, and the absence of D-periodicity arises when the lateral assembly of the molecules is distorted.
This, combined with our results, would imply that piezoelectricity in type I collagen fibrils does not arise solely from the hexagonal packing of collagen molecules. Our results are consistent with previous studies showing non-fibrillar collagen is piezoelectric\textsuperscript{11} and more recently that one of the amino acids present in collagen, namely, glycine, is piezoelectric.\textsuperscript{27} The measured shear piezoresponse of the non D-periodic, aligned type I collagen fibril membranes (~0.1 pm/V when calibrated via the lateral geometric method as described in section 2.2.3.2) is roughly an order of magnitude lower than the local and macroscopic shear piezoresponse of D-periodic type I collagen fibrils within rat tail and bovine Achilles tendons (~1-2 pm/V).\textsuperscript{8,10,28} The shear coefficient measured via PFM for D-periodic reconstituted collagen fibrils was 1.6 pm/V (calibrated data from ref. 10). The reduction in the piezoelectric signal of the membranes therefore may be attributable to a lower degree of ordered molecular assembly, as evidenced by the lack of D-periodicity in the membranes, than is present in D-periodic collagen fibrils. The polar orientation of type I collagen fibrils is also retained in the opaque membrane, as seen in Fig. 4c as a 180° phase shift between opposing domains. The AFM height image of a 5 × 5 µm\textsuperscript{2} area (Fig. 4.6 (d)) compared with the LPFM amplitude image in Fig. 4.6 (e) reveals that each piezoelectric domain contains only ~1-3 fibrils. There are discontinuities in the collagen membrane, as highlighted by black arrows in Fig. 4.6 (d), which results in a low piezoelectric signal (shown in Fig. 4.6 (e)). After neutralization, the topography of the opaque membrane appears altered (Fig. 4.7 (a)).
Figure 4.7 LPFM images of opaque collagen membrane after the neutralization process. (a) AFM height image displaying non D-periodic collagen fibrils (z-scale = 560 nm; scale bar = 2 µm). (b) LPFM amplitude image of same area in (a) displaying retention of piezoelectric domains as seen before neutralization. (c) LPFM phase image mapping the polarity of domains in (b). (d) AFM height image (z-scale = 200 nm; scale bar = 1 µm). (e) LPFM amplitude image of area shown in (d) and corresponding (f) LPFM phase image displaying polarity of domains. Reprinted from [18].

The fibrils have the same direction of alignment and do not exhibit the crimped structure visible in the *transparent* membrane. The measured width of the fibrils increased to 412 ± 51 nm, indicating a possible swelling of the fibrils, but this increase in fibril width is not statistically relevant. The LPFM amplitude image (Fig. 4.7 (b)) reveals that the domain structure of the *opaque* membrane has also remained unchanged after neutralization. The averaged piezoresponse for 10 domains is 0.775 ± 0.058 a.u., which is only ~ 1.1 fold than that measured before neutralization, indicating that the change of pH does not affect the piezoelectric properties of the *opaque* membrane as significantly as it
does for the transparent membrane. The crimped collagen structure obtained in the transparent membrane is formed by combining left-handed and right-handed helical-like arrays of fibrils into a double super-helix structure [34], whereas the opaque membrane has only a nematic orientation of helical-like arrays of fibrils. The presence of left-handed fibrils was also confirmed in rat tail tendon and ligament fibrillar crimps. Thus, compared to the opaque membrane, the transparent membrane has an additional translational order in the direction perpendicular to the fibril alignment. Another significant difference between the two membranes is the larger fibrillar width present in the opaque membrane compared to that of the transparent membrane. The dependence of the piezoelectric response on the pH of collagen in the transparent membrane could therefore be attributable to a size or symmetry effect, the full elucidation of which would require further study beyond the scope of this work.

PFM measurements observed that there is a 1.35 fold increase in the lateral piezoresponse of the transparent membranes containing collagen nanofibrils when the pH is adjusted from acidic (pH = 2.5) to neutral (pH = 7) conditions. This increase in piezoresponse could be due to an increased activity of the polar bonds of collagen at neutral pH and further corresponds to a structural change in the membrane, namely the formation of a crimp structure. Since it has been shown that collagen structure can greatly affect cell behaviour (Fig 4.1), the change in piezoelectric properties of collagen with pH and the presence of piezoelectricity in general, may also contribute to the affected cell behaviour. In addition, shear piezoelectricity was observed in non D-periodic collagen fibrils. These results may force the re-evaluation of how piezoelectricity manifests in collagen. Piezoelectricity is generally attributed to the hexagonal packing of monomers
within a collagen fibril. In the case of non D-periodic fibrils, this structure may not be accurate due to the incorrect lateral assembly of collagen molecules. Adjusting from acidic to physiological pH did not significantly change the lateral piezoresponse of the opaque membranes. The piezoelectric response has also been shown to be sensitive to whether collagen molecules are correctly assembled within a fibril. Interestingly, many diseases associated with collagen (e.g., osteogenesis imperfecta) are associated with the replacement of glycine in the collagen triple-helix with a larger amino acid. This deforms the triple helix, affecting the structure and subsequent fibril assembly. In the case of collagen from femora in Brtl mice (model for osteogenesis imperfecta), a larger variation in the periodicity was found in comparison to collagen from healthy mice. Thus, AFM and PFM may become a useful tool for distinguishing between, e.g., healthy and damaged or diseased tissue.
4.4 References


Chapter 5

Evaluation of electromechanical properties of assembled type I collagenous tissues

5.1 Introduction

The polar architecture was revealed in natural tissues via PFM in chapter 3, which will be relevant for further studies regarding the role of polarity in tissue growth. Yet in the area of tissue engineering, this property may not be adequately addressed as was highlighted in chapter 4. Collagenous tissues comprise a major constituent of the extracellular matrix, providing structural support for cells, and performing important developmental and physiological functions.¹ The tissue-specific orientations of collagen fibrils give rise to different functional and mechanical properties, which are difficult to replicate in vitro.²
Previous studies have demonstrated the potential of collagenous scaffolds and hydrogels for tissue engineering applications. The ability to assemble collagenous scaffolds in vitro with the same structure and properties as those formed by tissue collagen would be of significant importance for studying cell-matrix interactions as well as for developing compatible engineered tissues. In addition, comparing the electromechanical properties between natural collagenous tissues and engineering collagenous constructs could help understand the biological significance of piezoelectricity in collagen. Numerous approaches to assemble fibrillar collagen structures have been implemented, including hydrodynamic flow in the presence of potassium, magnetic field alignment, dip-pen lithography, chemical nanopatterning, microfluidics and AFM manipulation. Recent attempts to align collagen by electrochemical processes have demonstrated successful alignment of anisotropically oriented collagen monomers. In addition to advantages such as increased mechanical stability, aligned collagenous tissues have been shown to influence cell alignment and growth.

Cells have been shown to co-align with aligned collagen fibrils, suggesting that ordered fibrils influence cell polarization. It has also been shown that cells align in the direction of mechanical loading in hydrogels, which has been attributed to the triggering of cell-surface stretch receptors by mechanical signals. It seems plausible to suggest that such mechanical signals could transform into electrical signals as a result of collagen piezoelectricity, as suggested in section 1.2.3. Previous studies have also shown that electrical signals of various strengths and pulses lead to a significant increase in bone cell proliferation.
Successfully assembling collagen-rich tissues with similar alignment, orientation, and piezoelectric properties as natural tissues will provide a framework to further our understanding of the role collagen structure and function has on intercellular and cell-matrix communication. There may be unidentified benefits of replicating both functional and structural properties of collagenous tissues. By replicating the phenomenon of electromechanical coupling in assembled tissues, the applications of engineered collagen structures can be expanded. Notably, electromechanical coupling is a phenomenon exhibited by many biopolymers, including chitin, cellulose, poly-L-lysine, etc., which are currently used for biomedical applications. Piezoelectric polymers have also been shown to promote neurite alignment. It is envisioned that not just the structural and chemical properties of such biomaterials should be tailored, but so should the electromechanical properties, in order to elicit desired outcomes for targeted applications.

Here, a comparison of the electromechanical properties of iso-electrically focused collagen with rat tail tendon is presented. Rat tail tendon is an excellent tissue for studying collagen as it consists primarily of type I collagen, with only a small volume of proteoglycan, in which the fibrils have a high degree of alignment along the tendon axis. Similarly, iso-electric focusing has been shown to induce the conformational alignment of collagen monomers, which then assemble into hierarchical structures (nano- and micro-fibrils) that co-align along axis of the hydrogels.
5.2 Materials and methods

5.2.1 Preparation of rat tail tendon

Tendon harvested from a rat tail was deposited on gold-coated mica and fixed via carbon tape. The tendon fiber used for the study is $440 \pm 20 \mu m$ in diameter and $5.20 \pm 0.02 mm$ in length, measured via a micrometer. The tendon has been bleached in a 4% sodium hypochlorite solution for 20 s in order to partially remove non-collagenous proteins, and thereby expose the fibrils.

5.2.2 Preparation of iso-electrically focused collagen

Self-assembled, iso-electrically focused collagen hydrogels have been prepared via a method described previously by Abu-Rub et al.\textsuperscript{4}

![Illustration of iso-electric focusing method to produce an aligned collagen hydrogel. Reprinted from [4].](image)

Figure 5.1 Illustration of iso-electric focusing method to produce an aligned collagen hydrogel. Reprinted from [4].
A solution of dialyzed type I collagen monomers in a 20 nM acetic acid solution is subjected to a DC voltage of 3 V at a current of 25 µA in an electrochemical cell for 60 min, as shown schematically in Fig. 5.1. This generates a pH gradient between the electrodes which causes the collagen monomers to migrate towards their isoelectric point (pH of ~8). The monomers concentrate and subsequently assemble along this region to form a dense fiber bundle, which is then removed and incubated in polyethylene glycol (PEG)-containing buffer at 37 °C prior to incubation in phosphate buffered saline (PBS) overnight. The collagen hydrogel is then placed on highly ordered pyrolytic graphite and allowed to dry in air prior to PFM measurements. The dried collagen hydrogel used in the study is 500 ± 20 µm in diameter and 6.50 ± 0.02 mm in length.

5.2.3 Atomic force microscopy and piezoresponse force microscopy experiments

PFM imaging has been performed with an Asylum Research MFP-3D AFM equipped with a Zurich Instruments HF2LI lock-in amplifier and using conductive MikroMasch DPE18 Pt-coated cantilevers with nominal resonant frequencies and spring constants of 75 kHz and 3.5 N/m, respectively. The cantilever stiffness is expected to minimize any electrostatic contribution to the measured signal. Prior to imaging, the rat tail tendon and iso-electrically focused collagen samples are placed on a grounded copper plate. During imaging, an AC signal (typically 30 V\text{rms} at 7 kHz) is applied to the tip, which is in contact with the surface at a constant force (typically 90 nN). The shear sensitivity was calculated based on the geometry of the cantilever, as described by Peter et al. and is described in more detail in section 2.2.3.2. The out-of-plane sensitivity was
experimentally measured via the force curve method (53.6 nm/V). Therefore the in-plane sensitivity is 913 nm/V.

Fibril widths have been estimated from the AFM line profiles of ten fibrils. AFM deflection images are used as individual fibrils are visible for both the dried hydrogel and tendon in these images. For each fibril width reported, three line profiles are taken along the length of the fibril to account for any non-uniform fibrillar widths. Only fibrils which were visibly isolated were chosen for analysis.

5.2.4 Fast Fourier transform analysis

Fast Fourier transform (FFT) analysis (WSxM)\textsuperscript{24} has been used to characterize the alignment of collagen fibrils from both rat tail tendon and iso-electrically focused collagen. By conducting FFT analysis on an image, the information in the image is converted into frequency space. The subsequent FFT output image contains pixels which are distributed in a shape which represents the degree of alignment in the original image. In general, the more symmetric the shape is, the higher the degree of alignment which is present in the image. Direct comparison of the FFTs from rat tendon and the dried collagen hydrogel is possible using a radial profile plug-in in ImageJ. By summing the pixel intensities along the radius of the circular projection between 0° and 360° in 1° increments, the FFT distributions can be visualized and analyzed quantitatively.
5.3 Results and Discussion

5.3.1 AFM characterization of rat tail tendon and iso-electrically focused collagen hydrogel

Surface properties of rat tail tendon have been characterized via AFM. An AFM deflection image of a 5 x 5 µm² area of the tendon is shown in Fig. 5.2 (a). A periodic banding of 62.5 ± 0.4 nm is observed, as measured by a sine fit of the line profile shown in Fig. 5.2 (c). This periodicity is more readily apparent in the deflection as opposed to height images. The fibrils appear to be predominately oriented along the long axis of the tendon. The average observed fibril width has been determined to be 232 ± 35 nm, calculated from an average of ten fibrils in the image, which is within the range reported for rat tail tendon (typical fibril diameters are between 50 nm and 300 nm and vary with the age of the rat²²).

Fig. 5.2 (b) shows an AFM deflection image of the dried collagen hydrogel. Several fibrils are aligned along the length of the hydrogel axis, but a considerable number of misaligned fibrils remain. The line profile in Fig. 5.2 (d) and sine fit resulted in a calculation of the D-periodicity of 66.7 ± 0.2 nm of collagen and a typical fibril width of 227 ± 46 nm was calculated from an average of 10 fibrils in the image. These results demonstrate the successful replication of fibrillar type I collagen with similar fibril widths. However, from this image, we see this particular collagen hydrogel does not have the same degree of fibrillar alignment as the rat tail tendon. Note that the PEG buffer stimulates volume reduction by dehydration, which assists with the fibrillar alignment,
Assembled collagenous tissues

thus the tendon and hydrogel samples likely have different water contents and may deform differently during sample preparation.

Figure 5.2 AFM images of rat tail tendon and iso-electrically focused hydrogel. (a) AFM deflection image of a rat tail tendon. (b) AFM deflection image of iso-electrically focused collagen. (c) Line profile measured from (a) displaying the periodicity of the collagen fibrils. (d) Line profile measured from (b) confirming collagen periodicity in iso-electrically focused collagen fibrils. Scale bar for (a) and (b) is 1 µm. Reprinted from [25].

5.3.2 PFM characterization of rat tail tendon and iso-electrically focused collagen hydrogel

As collagen is a shear piezoelectric biopolymer,\textsuperscript{26-28} LPFM has been applied to investigate the in-plane piezoelectric response of the tendon and dried hydrogel. A 20 x
20 µm² area deflection image of the rat tail tendon is shown in Fig. 5.3 (a). At this scan size, there is no periodic banding visible. Note that in some regions, the tendon may not have been bleached sufficiently to reveal the fibrils. PFM, however, probes a finite volume beneath the tip (as demonstrated in chapter 3) such that the response of the fibrils underneath can still be visualized. Fig. 5.3 (b) is the corresponding LPFM amplitude image, which confirms piezoelectricity in the tendon. Each fibril appears to have a constant piezoresponse value along the length of the fibril, and the image illustrates a high degree of alignment of the fibrils.

Figure 5.3 PFM images of rat tail tendon. (a) AFM deflection image of a rat tail tendon. The inset shows the cantilever orientation and scanning direction (double arrow). (b) LPFM amplitude image measured in the same region as (a). (c) LPFM phase image displaying the polar orientation of the fibrils. Scale bar for (a–c) is 5 µm. (d) Smaller scan size AFM image measured from the location indicated by the square in (b), which shows the periodicity of the fibrils. (e) LPFM amplitude and (f) LPFM phase images of the same
region as (d). Arrows in (f) indicate the assigned collagen polarity from N to C termini. Scale bar for (d–f) is 1 µm. Reprinted from [25].

The LPFM phase image (Fig. 5.3 (c)) displays the N to C polarity, or the polar order, of the collagen fibrils. Since it is non-trivial to identify the polarization direction, a fibril with bright phase contrast (+90°) is assigned as having a N to C polarity pointing to the right of the image, while a fibril with dark phase contrast (-90°) has a polar order pointing to the left. In essence, the fibrils are deforming out of phase with each other during the application of the AC bias. Note that there is clear evidence of anti-parallel polar ordering of the fibrils, whereby adjacent fibrils exhibit polarization in opposite directions. Fig. 5.3, (d-f) shows LPFM images obtained from a smaller area (5 x 5 µm² scan). Collagen fibrils are visible in the deflection image (Fig. 5.3 (d)), as confirmed by the presence of D-periodicity. The LPFM amplitude image (Fig. 5.3 (e)) displays fibrillar-level response consistent with the shear piezoelectricity of type I collagen. The average width of the fibrils in the LPFM amplitude image is 200 ± 78 nm. This suggests the response is due to the piezoelectric activity of individual fibrils as the average fibrillar widths from the AFM deflection image have been measured to be 232 ± 35 nm. From the LPFM phase image (Fig. 5.3 (f)), there is further evidence of anti-parallel fibrillar ordering along the tendon axis. By comparing the LPFM phase and amplitude images, it can be observed that fibrils which exhibit opposite polarization directions undergo equal shear piezoelectric deformations, as expected.
Figure 5.4 PFM images of iso-electrically focused collagen hydrogel. (a) AFM deflection image of iso-electrically focused collagen. The inset shows the cantilever orientation and scanning direction (double arrow). (b) LPFM amplitude image of same area as (a) and simultaneously recorded (c) LPFM phase image. Scale bar for (a–c) is 5 µm. (d) Smaller scan size AFM deflection image of iso-electrically focused collagen measured from the location indicated by the square in (b). (e) LPFM amplitude and (f) LPFM phase images. The regions circled in (e) show (1) a fibril aligned with the scan direction, and (2) a fibril oriented at an angle with respect to the scan direction. Arrows in (f) indicate the assigned collagen polarity from N to C termini. Scale bar for (d–f) is 1 µm. Reprinted from [25].

Lateral PFM has been utilized to study electromechanical coupling in dried iso-electrically focused collagen hydrogels to compare the shear piezoelectricity of natural and engineered collagen. Fig. 5.4 shows the LPFM results from the iso-electric collagen. Fibrils are visible throughout the entire deflection image in Fig. 3a. From the LPFM amplitude image in Fig. 5.4 (b), piezoelectricity is confirmed in the dried hydrogel,
suggesting a successful replication of this functional property. In the LPFM amplitude image, higher piezoresponse is observed from fibrils perpendicular to the cantilever and parallel to the scanning direction, which is due to the in-plane shear response of the fibrils and results from the torsional twisting of the cantilever. The cantilever-fibril geometry used for all PFM measurements is illustrated in the insets of Figs. 5.3 (a) and 5.4 (a). The long axis of the tendon and dried hydrogel sample is parallel to the scanning direction in all cases. The signal dependence on the angle between the cantilever and fibril is highlighted in Fig. 5.4 (e). Fibril 1 has the optimal orientation for measuring the shear response of the fibrils as it is perpendicular to the cantilever and parallel to the scan direction. Fibril 2 displays approximately half of the maximal response as it has a 37° angle with respect to the scan direction. LPFM amplitude images for rat tail tendon and the dried collagen hydrogel have been normalized to allow for direct comparison of both. From the calculated average piezoresponse from the amplitude images measured on both samples, the collagen hydrogel has a 35% higher signal than the tendon. There is also a higher response from fibrils with optimal orientation (perpendicular to the cantilever) in the dried collagen hydrogel when compared to similar fibrils in the rat tail tendon. This unexpected result may be attributed to incomplete bleaching (Fig. 5.2 (a)) of the tendon sample or to a change in the tip state, i.e., contamination or coating wear. However, it should also be noted that while the tendon collagen fibrils are cross-linked, there is no specific cross-linking step in the preparation of the hydrogel (noncovalent cross-links are likely present in the hydrogel). This would likely result in a larger piezoelectric signal due to a higher degree of freedom of the monomers, but would require an in-depth study. LPFM phase image shown in Fig. 5.4 (c) displays the polar order of the fibrils where a
+90° phase is observed for 81% of the image, illustrating that the anti-parallel polar ordering observed in the tendon is not replicated in the dried hydrogel.

5.3.3 Alignment studies of rat tail tendon and iso-electrically focused collagen hydrogel via FFT analysis

In Fig. 5.5, the degree of alignment of the fibrils in the tendon is investigated. The fibrillar alignment in tendon and iso-electrically focused collagen has been determined using FFT analysis of the PFM amplitude images and subsequent radial summation of pixel intensities. PFM amplitude images were chosen for the FFT analysis (as opposed to topography images) as the collagen fibrils are not fully exposed in the rat tendon case. FFT and radial averaging analysis of the phase and reconstructed $A_1 \sin (\omega t + \Phi)$ images follow the same trend demonstrated with the amplitude images. The FFT of an image containing aligned fibrils will yield an elliptical distribution. The degree of alignment is represented by the full width at half maximum (FWHM) of the peaks obtained from summing the pixel intensities of the FFT image for each degree between 0° and 360°.

Fig. 5.5 (a) shows the FFT of Fig. 5.3 (b), which has a narrow peak illustrating the alignment of the collagen fibrils in rat tail tendon. The radial summation of the intensity of the rat tail tendon FFT image (Fig. 5.5 (c)) reveals two peaks at 95° and 275°, indicating strong alignment along the scan direction, and hence, along the tendon axis. The slight offset from 90° and 270° is due to a slight misalignment of the scan direction with respect to the tendon axis. The FWHM of the peaks has been determined to be 34° and 42°, respectively. The radially-averaged intensity plots have been normalized using a scaling factor of 3300 to allow for direct comparison between tissues.
Figure 5.5 FFT analysis of rat tail tendon and iso-electrically focused hydrogel. (a) FFT of the rat tail tendon PFM amplitude image (Fig. 5.3 (b)) displaying the fibrillar alignment of natural tissue. (b) FFT of the iso-electrically focused collagen PFM amplitude image (Fig. 5.4 (b)) displaying alignment of grown fibrils. (c) Radial average intensity plot of the rat tail tendon FFT and iso-electrically focused FFT images. Reprinted from [25].

The alignment of collagen fibrils in the dried iso-electrically focused collagen hydrogel has also been studied using FFT analysis (Fig. 5.5 (b)). From the radial summation of the FFT image (Fig. 5.5 (c)), there are peaks at 85° and 275°, and the FWHM is measured to be 95° and 135°, respectively. Comparing the average FWHM
value for the tendon and dried hydrogel (38° and 115°, respectively), it is apparent that the collagen fibrils in the tendon are three times as well-aligned as those in the hydrogel.

It has been shown that the dried collagen hydrogel successfully replicates the characteristic D-period of natural type I fibrillar collagen. PFM images measured on the dried collagen hydrogel verify the piezoelectricity of the engineered tissue, demonstrating that iso-electrically focused collagen has similar piezoelectric properties as natural collagen. Thus, the results obtained demonstrate that iso-electrically focused collagen has similar structural and electromechanical properties to that of natural tendon. The observed higher piezoresponse signal in the dried hydrogel to that of tendon is thought to emanate from the lack of covalent crosslinks present in the dried hydrogel. More study is required on the role of covalent crosslinks in the piezoelectricity of biosystems. This study will provide a framework for further studies on cell-matrix communication and engineering tissues for mimicking, improving, and replacing biological functions.
5.4 References


Assembled collagenous tissues


Chapter 6

Evaluation of the electro-mechanical properties of individual type II collagen fibrils

6.1 Introduction

In chapters 3 – 5, we investigated the electromechanical properties of many forms of collagen type I via PFM, from assembled tissues to natural tissues down to individual collagen fibrils. There are, however, other collagen types yet to be investigated for these properties. Electromechanical phenomena in biological systems have been observed in other important biological processes not only in collagenous tissues\(^1\), but also in the form of voltage-controlled ion channels\(^2\), neurons\(^3\), muscle contraction\(^4\), lipid membranes\(^5, 6\), etc., and each are critical to the functionality of biosystems. While the link between
charge and cellular interactions is not well understood, previous studies investigating cellular adhesion and growth on poled hydroxyapatite suggest that surface charge influences cellular response.\textsuperscript{7}

The piezoelectric properties of collagen type I have been studied at scales ranging from the macroscale\textsuperscript{8} to, more recently, the nanoscale\textsuperscript{9} with the advent of PFM.\textsuperscript{10, 11} Collagen type I is a shear piezoelectric with a polarization along the fibril length (corresponding to N to C polarity as described in section 1.1.1).\textsuperscript{12} Other collagens, such as collagen type II (cartilage is 50 – 80\% type II of the dry weight\textsuperscript{13} and fibrils are typically 20 – 200 nm in diameter) have yet to be fully characterized for their nanoscale electromechanical properties. Perhaps such measurements have been overlooked since cartilage is avascular with few chondrocytes (comprising 2\% of the total volume of articular cartilage\textsuperscript{14}) and is thus unable to repair itself naturally,\textsuperscript{15} thereby implying an absence of biofunctional relevance for piezoelectricity in such tissues (but not an absence of piezoelectricity). However, the inability of the tissue to self-heal necessitates research efforts to understand tissue development and approaches for repairing or replacing it,\textsuperscript{16} which typically involve piezoelectric biomaterials.\textsuperscript{17} Therefore, there may be underutilized benefits of the presence of piezoelectricity in tissues such as cartilage.\textsuperscript{18}

In the case of articular cartilage, previous studies have shown the use of pulsed electromagnetic fields to stimulate proteoglycan synthesis and chondrocyte proliferation.\textsuperscript{16, 19-21} In fact, piezoelectric polymer membranes with alternating layers of chondrocytes (shown in Fig. 6.1) have been shown to result in a significant increase in the expression of relevant cartilage genes (fibronectin, collagen type II, SOX 9 and integrin α10) when compared to membranes consisting of only chondrocytes.\textsuperscript{17}
In addition, electromechanical behavior in the form of electrokinetics and electrostatics was demonstrated in cartilage previously, showing these phenomena play an integral role in the mechanical properties of cartilage. Since earlier studies of similar phenomena in bone lead to the suggestion that piezoelectricity can complement or even enhance the remodeling mechanism, it is reasonable to suggest piezoelectrically-induced charge might also have a biofunctional role in cartilage or other collagen type II-rich tissues. One prior study reported electromechanical response from canine femoral cartilage, but the piezoelectric coefficient was not determined, nor was the response proven to originate from type II fibrils. By investigating individual, isolated collagen type II fibrils, the challenge of quantifying the piezoelectric response which results from ‘bulk’ effects present in tissues is avoided. This phenomenon is discussed in more detail for the case of tendon in chapter 8.
Possible variances in the magnitude of the piezoelectric coefficients between collagen types I and II could be attributable to differences between collagen types in their molecular structure, the sequence of amino acid residues present, cross-linking, and dielectric properties. Given the prevalence of piezoelectricity in biomaterials, from polar bonds in tissues, to individual molecules, down to individual amino acids (such as glycine), many types and forms (e.g., fibrils and molecules) of collagen are likely piezoelectric.

Both collagen types I and II consist of three left-handed polypeptide (α) chains wound in a right-handed helix. Collagen type I is heterotypic, having two α1 chains and one α2 chain, while collagen type II is homotrimeric, comprising three identical α1 chains, as shown in Figure 6.2. Another difference in the structure of collagen type I and II is the difference in the type and number of crosslinks bonded between molecules. Collagen type II tissues such as cartilage have twice the number of crosslinks per unit collagen than that of type I tissues such as tendon.
Figure 6.2 Cross section of the type I and II collagen molecule (a) type I collagen molecule comprising two $\alpha_1$ and one $\alpha_2$ chains and (b) type II collagen molecule comprising three $\alpha_1$ chains.

Both collagen types form D-periodic fibrils with non-centrosymmetric crystalline cross sections leading to our hypothesis that collagen type II, like type I, is a shear piezoelectric material. Here, we use PFM to characterize the piezoelectric properties of collagen type II fibrils from chicken sternum cartilage in comparison with the properties of collagen type I fibrils from bovine Achilles tendon.

6.2 Materials and methods

6.2.1 Preparation of collagen type II and type I fibrils

Collagen type II from chicken sternum cartilage (Sigma Aldrich, C9301) was dissolved in 40 mM acetic acid at 4°C overnight. The resulting collagen solution was added to a buffer of 50 mM glycine and 200 mM potassium chloride adjusted to 9.2 pH to make a final collagen solution with a concentration of 300 $\mu$g/mL. 100 $\mu$L of the resulting
collagen solution was placed on a glass slide, to which collagen adheres well, and air dried.

Collagen type I from bovine Achilles tendon (Sigma Aldrich, C9879) was swollen in 0.01 M hydrochloric acid at 0°C overnight. The resulting solution was shredded using a blender (Braun, MR 400 HC) for 10 minutes and diluted in phosphate buffered saline to a concentration of 100 µg/mL. 100 µL of the solution was pipetted on a glass slide and incubated for 10 minutes before rinsing in ultrapure water (Millipore, Gradient A10, 18.2 MΩ·cm) to avoid salt crystal formation. The sample was placed under a gentle stream of nitrogen and subsequently air dried.

6.2.2 PFM measurements

During PFM measurements, a conductive cantilever (CSC37 (tip B), Mikromasch) was in contact with the surface (typical force applied ~ 20 nN) and an AC voltage (typically 20 V at 10 kHz) was applied. The applied voltage results in a bias-induced shear surface deformation, detected as the lateral signal from the photodetector, which is demodulated into amplitude, $R$, and phase, $\theta$, via the lock-in amplifier. $R$ and $\theta$ are sensitive to the magnitude of piezoelectric deformation and the local polar orientation, respectively. AFM topography images were 1st order flattened, while PFM images were not flattened.

6.3 Results and discussion

6.3.1 Structural characterization of collagen type II fibrils via AFM

Topographical properties of collagen type II fibrils were examined using contact mode AFM. A 3D AFM topography image comprising individual type II fibrils is shown in Fig.
6.3. Collagen type II is known to exhibit the established 67 nm D-periodicity associated with collagen type I, as seen in Fig. 6.3 (a). Fibrils were found to be ~ 6 ± 4 µm in length and ~ 740 ± 185 nm in diameter, measured via line profiles (n = 12). Every fibril studied had tapered ends, a shape associated with reconstituted collagen type II. An example of a line profile from a type II fibril is displayed in Fig. 6.3 (b). A polynomial fit was used to subtract the background topography, and a sine fit was used to determine a D-periodic spacing (or inverse frequency) of 66.7 ± 0.1 nm.

Figure 6.3 (a) AFM topography image of collagen type II fibrils. The z-scale is 520 nm. (b) Typical line profile showing characteristic D-periodicity of collagen type II. Solid grey line is a section profile from (a) (background subtracted) and the solid black line is a sine function fit, which is used to measure the D-periodicity. Reprinted from [36].
6.3.2 *Investigating the presence of piezoelectricity in collagen type II fibrils*

The electromechanical properties of type II fibrils were investigated via PFM. The molecular packing in type II fibrils is similar to that for type I fibrils, suggesting they will also behave as shear piezoelectric materials. Thus, LPFM was used to measure the shear piezoelectric response of the fibrils. A 35 × 35 μm AFM topography image of type II fibrils is shown in Fig. 6.4 (a). Shear piezoelectricity is confirmed in the LPFM mixed piezoresponse image \((R \cdot \cos \theta)\) (Fig. 6.4 (b)) of fibrils in the same area as Fig. 6.4 (a). The mixed LPFM image contains information both on piezoelectric magnitude (a.u.) and the sign of the piezoelectric coefficient (from the PFM phase data). The color represents the direction of polarity along the length of the fibrils, with different colored fibrils having opposite directions of polarity.

Figure 6.4 PFM study of collagen type II fibrils (a) AFM topography image displaying several collagen type II fibrils. The z-scale is 300 nm. The cantilever indicates the fibril-
cantilever geometry during the experiment. (b) Mixed piezoresponse image of the same area shown in (a). The z-scale is 1.2 a.u. Bright and dark fibrils represent opposite polarization orientations, directed from N to C termini. Reprinted from [36].

Given the structural similarities with type I, it is expected that a type II fibril oriented with its longitudinal axis perpendicular to the cantilever axis (in the laboratory x-y plane) will exhibit a maximum shear response. The magnitude of piezoelectric response is indicated by the ‘brightness’ of the fibril along its axis. The cantilever orientation is depicted via the inset in Fig. 6.4 (a). This is confirmed by comparing a fibril with this orientation to a fibril with an angle of less than 90° between the fibril and cantilever axis.

6.3.3 Comparison of the electromechanical properties between collagen type II and type I fibrils

Quantitative PFM remains a challenge; therefore the relative shear coefficients were measured and compared for collagen type II and collagen type I using the same tip and PFM conditions.
Figure 6.5 Comparison of piezoelectric properties between collagen type I and II. 3D AFM topography image of (a) type I and (b) type II fibrils with mixed piezoresponse overlays. The z-scale is 90 nm for (a) and 180 nm for (b). The visible D-periodicity is present in the topography data only. (c) Averaged line profiles \( (n = 200, \text{background subtracted}) \) taken from the mixed piezoresponse data in ((a) red) and ((b) blue). (d) Weighted mean of PFM amplitude as a function of AC voltage (background subtracted) recorded from locations on fibrils in ((a) red \( (n = 5) \)) and ((b) blue \( (n = 10) \)). Reprinted from [36].

Fig. 6.5 (a) and (b) display 3D AFM topography images of collagen type I and II, respectively, with their \( d_{15}^0 \) piezoelectric coefficient maps overlaid. Comparison of the averaged line profile \( (n = 200) \) of the piezoresponse of both images, as shown in Fig. 6.5
Type II collagen fibrils

(c), confirm that the response of collagen type II is less than that of type I. In order to further quantify this difference, PFM amplitude as a function applied AC voltage was measured in several locations, whereby the tip was placed in constant contact with the fibril. Determination of the slope of the resulting graph yields the effective piezoelectric coefficient. A linear increase in PFM amplitude as a function of voltage is not evident until after 10 V, as shown in Fig. 6.5 (d). This may be due to the signal being smaller than the noise floor of the instrument when small voltages are applied. Hence, the slope was determined between 10 – 30 V in order to more accurately represent the linear piezoelectric regime. Fig. 6.5 (d) shows the results of the representative point measurements recorded on type I and type II fibrils, further illustrating the reduced effective piezoelectric coefficient of collagen type II. In fact, determination of the slope and linear fit error show that the average shear coefficient for type I (2.2 ± 0.5 a.u., n = 5) is roughly 68% higher than that of type II (0.7 ± 0.2 a.u., n = 10). The increase in piezoelectric signal for type I cannot be attributed to tip wear, etc. as type II was investigated prior to type I using the same tip. Type I collagen was also prepared using the type II protocol to ensure the preparation methods used were not responsible for the differences observed. Values measured on type I fibrils prepared via this preparation method were 2.5 ± 0.7 a.u. (n = 3). The piezoresponse for both preparations is statistically equivalent, showing in this case that the sample preparation is not a factor involved in the differences observed.

When the piezoelectric signals are calibrated via geometric scaling as described in section 2.2.3.2, the measured shear coefficient for type I is in the same range as previously reported with PFM. Based on a recent lateral calibration method where the
lateral signal is measured as a function of surface tilt (also described in section 2.2.3.2),
the measured shear coefficient is 0.15 ± 0.03 pm/V for type I and 0.05 ± 0.02 pm/V for
type II. This is an order of magnitude lower than the shear coefficient when calibrated
using the geometric method. More research is needed in the lateral PFM community to
discuss the disparity between calibrations.

6.3.4 Cantilever-fibril angle dependence of collagen type II fibrils

For the C₆ class symmetry of D-periodic fibrillar collagens,¹ ³⁹ the shear coefficient will
depend on the angle between the cantilever and fibril axis.³⁹ In order to determine if
collagen type II has angle-dependent shear piezoelectric properties similar to collagen
type I, the piezoelectric coefficients were determined from mixed piezoresponse LPFM
images of 17 fibrils with similar diameters (~ 700 nm) but different orientations with
respect to the cantilever (shown in Fig. 6.6 as a function of angle between cantilever and
fibril).

Figure 6.6 Shear mixed piezoresponse measured as a function of cantilever-fibril angle.
Solid black line represents a sine fit, demonstrating the angle dependence of the shear
piezoresponse. Reprinted from [36].
In this case, the piezoelectric coefficients were determined as the difference in the mixed piezoresponse signal between the fibril and substrate. The average and standard deviation of the piezoresponse was calculated using $300 \times 300$ nm regions on each fibril (Igor Pro, Wavemetrics). The background signal, likely originating from electronic offsets, mechanical resonances and/or electrostatic interactions, was also determined from a $300 \times 300$ nm region and then was subsequently subtracted from the measured fibril piezoresponse to give the value reported. Note that while the background signal may appear as an offset in the effective piezoelectric coefficient measurement, it does not affect the values reported, which depend only on the slope. Fig. 6.6 shows a maximum in the mixed piezoresponse signal when the cantilever is orthogonal to the fibril, as expected, and the data follows a sine dependency as highlighted by the fitted sine function (solid black line).

Possible explanations for the different piezoresponse measured for collagen types I and II include the use of different sources and the different polypeptide chains between the types. In addition, the difference could be related to the higher number of covalent cross-links present in type II vs. type I, which would lead to the fibril being more mechanically stable, resulting in reduced deformability. However, the density of cross-links is not quantified here and it is likely that the collagen source does not contain all native cross-links. Further investigations in the area of electromechanical coupling in biopolymers are required to better understand the influence of structure, source, location of tissue, etc. on their piezoelectric properties. This study highlights the importance of quantifying the electromechanical properties of different collagen types, source, etc., in order to better understand the manifestation of piezoelectricity in collagen and other
biosystems. In addition, the electromechanical properties reported here for collagen type II fibrils could inspire additional research into the area of cartilage repair since recent efforts in cartilage engineering center on the application of electromagnetic fields to increase chondrocyte proliferation and extracellular matrix synthesis.\(^\text{16}\) It is also possible that the piezoelectric-induced charge plays a role in other functionalities of the cartilage such as its low frictional properties, since charge plays a critical role in, e.g., bioinspired low friction polyelectrolyte brushes.\(^\text{41}\) Further studies are needed to understand the role piezoelectrically-induced charge plays in all collagenous tissues with an emphasis on its influence on tissue formation, growth, and repair.

Collagen type II has been shown via PFM to behave as a shear piezoelectric, exhibiting an angle dependence of the piezoelectric signal with cantilever-fibril angle. The shear piezoelectric coefficient of the type II fibrils studied was determined to be ~ 28 – 32% lower than that measured for collagen type I. A uniform polarization directed from the amine to carboxyl termini was observed in all fibrils studied. Explanations for the reduced piezoelectric coefficient for collagen type II are discussed and the lack of understanding of the role piezoelectrically-induced charge plays in biosystems is highlighted. The reported electromechanical properties of collagen type II fibrils may stimulate more research into the biofunctionality of piezoelectricity in type II rich tissues, such as cartilage.

### 6.4 References


Chapter 7

Determination of the piezoelectric tensor of collagen at the nanoscale

7.1 Introduction

In previous chapters 3 – 6, PFM has been demonstrated on several different collagenous structures. However, in order to relate piezoelectrically-induced charges for biofunctional processes, quantification of the piezoelectric tensor at the relevant length scale (i.e. at the nanoscale) would be ideal. The use of external electrical stimulation in bone to promote fracture healing dates from 1841, and continues to be used\textsuperscript{1-3} despite the underlying mechanism remaining elusive. Similarly, mechanical stimulation is known to have a pronounced effect on the rate of bone formation. Named after Julius Wolff and qualitatively accepted by clinicians, Wolff’s Law describes the ability of bone to adapt to mechanical loads via mechanotransduction.\textsuperscript{4} Thus, bone remodeling can be influenced by both electrical and mechanical stimuli. The electrical reaction of bone as a response to
Piezoelectric tensor of collagen

stress is described in more detail in sections 1.2.3 and 3.1. Electromechanical coupling in bone was first reported by Fukada and Yasuda\textsuperscript{5} who demonstrated that bone behaved as a classical piezoelectric material at the macroscopic scale with a piezoelectric tensor comprising only a \(d_{14}^0\) coefficient. Thereafter, the direct piezoelectric effect in bone was linked with the ability of bone to remodel.\textsuperscript{5} Fukada and Yasuda also reported that tendon, which comprises highly aligned collagen fibrils, is piezoelectric with a hexagonal \(C_6\) class symmetry,\textsuperscript{7} suggesting that the piezoelectricity of bone is due to the presence of collagen. Subsequent studies revealed that the principal contributor to piezoelectricity in bone is collagen.\textsuperscript{8} Piezoelectricity in collagen arises due to its symmetry lacking an inversion center (hexagonal as described above) with the collagen fibril comprising a uniform polarity directed from the amine to carboxyl termini along the fibril length, as described in more detail in section 1.2.2. Recent experiments, whereby hydroxyapatite (the mineral phase of bone) deposition occurred on cyclically deformed cortical bone collagen (the organic matrix of bone), suggest that piezoelectric generation of electric charge is a primary mechanism of bone remodeling.\textsuperscript{9}

The complex response of bone to a wide variety of forces (compressive, shear, axial, etc.) is currently poorly understood. Studies have shown the microstructure of collagen and Haversian systems are suited to resist these forces\textsuperscript{10}. The high degree of structural organization of collagen in bone and the tensorial nature of collagen piezoelectricity imply that the sign and magnitude of piezoelectrically-induced charge will depend on the location within the bone and the mechanical load. Thus, collagen piezoelectricity is uniquely suited as a means for cells to locally differentiate between mechanical loads of varying magnitude and direction. Large variations occur in the magnitude of the charge
exhibited locally across the surface of bone when a stress is applied\textsuperscript{11}, which was experimentally measured on dried femur as shown in Fig 7.1.\textsuperscript{12} Piezoelectricity is a promising explanation for this phenomenon.

Figure 7.1 Charge distribution (given in pC/cm\textsuperscript{2}) of bone when the medial surface is displaced (stressed). Reprinted from [12].

To date, however, the full piezoelectric tensors of tendon and bone have only been determined on the macroscale, while the crystal structure of collagen has been known\textsuperscript{13} and the tensor of collagen has been inferred from its single crystal equivalents and
macroscale measurements. By their nature, macroscopic piezoelectric measurements represent an average measurement of numerous collagen fibrils. For this reason, it is difficult to obtain accurate values of piezoelectric coefficients for an individual collagen fibril from a macroscopic measurement. In fact, as seen in section 3.3.2, nanoscale measurements of a tendon cross section revealed that while collagen fibrils were highly aligned, they did not have unipolar N to C polarities. Thus, the piezoelectric displacements from adjacent fibrils with opposite polarities may cancel each other out in macroscopic measurements. Therefore, macroscopic measurements will not accurately represent the properties of individual fibrils and the piezoelectric tensor of a single collagen fibril remains unknown.

Since cellular responses to electrical signals occur and are sensed at the local scale, as described above, determining an accurate piezoelectric tensor for collagen at the fibrillar scale will have implications for investigating and exploiting any associated biofunctionality of piezoelectricity in collagen. With the advent of PFM, it is now possible to probe piezoelectricity in biosystems, including collagen, with nanoscale resolution. PFM measurements of adult humerus and tibia bone under both dry and wet conditions revealed a nonzero longitudinal piezoelectric response ($d_{33}^0 \sim 8 \text{ pm/V}$). Macroscopically, however, a negligible longitudinal piezoelectric response was measured in bone, illustrating a disparity between local and macroscopic measurements. Similarly, limited longitudinal response ($d_{33}^0 = 0.08 \text{ pm/V}$) was measured macroscopically in tendon, yet local measurements again suggest a higher longitudinal signal. Nanoscale shear piezoelectric measurements on collagen, however, were of the same order of
magnitude as those seen macroscopically in tendon. These studies highlight the gap in our knowledge between the macro- and nanoscale piezoelectric properties of collagen.

Here, we present the piezoelectric tensor of collagen at the individual fibrillar level using PFM, thus attempting to bridge the knowledge gap between the macro- and nanoscale. Since collagen fibrils in bone are difficult to isolate due to the hierarchical structure of bone and the mineralization of the collagen, tendon, which often serves as a model for mineralization studies, is used for this study. Both LPFM and VPFM piezoresponse signals were measured for tendon sectioned at three different angles (0°, 45° and 90°) relative to the major axis of the tendon. These measurements (taken in the laboratory coordinate system) were related to the sample coordinate system in order to calculate the piezoelectric coefficients of collagen at the scale of an individual fibril.

7.2 Materials and methods

7.2.1 Sectioning of rat tail tendon

Tendon was harvested directly from the tail of a 4 week old rat. The tendon was then fixed in 4% paraformaldehyde for 1 hour prior to embedding in epoxy (Epon 812, Sigma Aldrich). The epoxy resin was cured over a 36 hour period at 45 °C, lower than the thermal denaturation temperature of rat tail tendon (64 °C). Three embedded tendons were trimmed and polished (280 and 1000 grit silicon carbide grinding paper at 200 – 400 rpm). 10 µm-thick sections were cut using a microtome (EM UC6, Leica) and subsequently imaged using PFM.
7.2.2 PFM measurements

For PFM measurements, typically 30 V was applied to the tip at a 10 kHz (LPFM) or 20 kHz (VPFM) modulation in contact with the surface (typical imaging force ~ 100 nN). The modulation frequency was chosen to be much lower than the contact resonance in order to avoid resonant enhancement of the measured signal (described in more detail in section 2.2.2.1) and to ensure consistent, quantitative results. The bias-induced surface deformations were demodulated into $X$ and $Y$ Cartesian signals ($X = R \cdot \cos \Phi$ and $Y = R \cdot \sin \Phi$) where $R$ is the amplitude of piezoelectric deformation and $\Phi$ is the phase difference between the excitation and the measured signal and contains information on polar ordering, which relates to the N to C polarity in the case of collagen.$^{14}$ PFM images were obtained in contact mode and presented as the $X$ Cartesian signal. Hence, the images presented contain information on both the amplitude of piezoelectric deformation and the local polar orientation of the collagen fibrils. Shear coefficients have been calibrated via a geometric lateral calibration based on the cantilever dimensions as described in section 2.2.3.2. The geometric dimensions of the levers used in the study have been directly measured using SEM, an example of which is shown in Fig. 7.2.
Figure 7.2 SEM image of cantilever used in the PFM study. The dimensions of which (cantilever length and tip height) were used in the calibration of shear coefficients.

7.2.3 Correlation analysis of various sections

In order to determine and compare the characteristic domain sizes between the 0°, 45°, and 90° sections, a 1 dimensional (1D) autocorrelation analysis was undertaken using the function:

$$G(k_1, k_2) = \sum f(x, y)f(x + k_1, y + k_2)$$  \hspace{1cm} (1)

where $k_1$ and $k_2$ are vectors shifted in the $x$ and $y$ axes, respectively. The characteristic size was determined from the normalized correlation function via:

$$G(k_1, k_2) = \sigma^2 e^{\frac{x}{{T^2}}}$$

where $T$ is the characteristic size along either the laboratory $x$ (or $y$) axis representing the width and length of the piezoelectric domains, respectively.
7.2.4 PFM point measurements

Single point measurements were conducted in order to determine the relevant piezoelectric coefficients, whereby the tip was placed in contact with the surface at several locations per sample (n = 20 per coefficient) with the amplitude (piezoelectric deformation) measured as a function of applied voltage. The piezoelectric coefficient (pm/V) was calculated from the slope of the graph. Calibration of the vertical optical lever sensitivity was obtained from the force curve method (described in section 2.2.3.1 and typically 70 ± 7.4 nm/V). A range of methods was investigated to calibrate the lateral optical lever sensitivity which are described in section 2.2.3.2, however, unless otherwise stated, then geometrical conversion is used (lateral InvOLS was typically 882 ± 7 nm/V). The geometrical dimensions were determined via SEM of the cantilevers used in this study.

7.3 Results and Discussion

7.3.1 Crystal orientation effects

To determine each piezoelectric coefficient for collagen, the relationship between the measured (laboratory) and sample coordinate system must be considered and solved for. For a known sample orientation, the laboratory coordinate system \((d_{ij})\) can be related to the sample coordinate system \((d^0_{kl})\) using the well-known relation:

\[
 d_{ij} = A d^0_{kl} N \quad (2)
\]

where \(A\) is a transformation matrix along 3 degrees of freedom \((\theta, \psi, \phi)\) corresponding to rotations around the \(x\), \(y\), and \(z\) axes, respectively, \(d^0_{kl}\) is the piezoelectric tensor for the
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sample coordinate system, and the Bond transformation matrix, \( N \). For tendon, with \( C_6 \) hexagonal symmetry, \( d_{31}^0 = d_{32}^0, d_{14}^0 = -d_{25}^0, \) and \( d_{15}^0 = d_{24}^0 \), which takes the form of:

\[
d_{kl}^0 = \begin{pmatrix}
0 & 0 & 0 & d_{14}^0 & d_{15}^0 & 0 \\
0 & 0 & 0 & d_{15}^0 & -d_{14}^0 & 0 \\
0 & d_{31}^0 & d_{33}^0 & 0 & 0 & 0
\end{pmatrix} (3)
\]

This simplifies the number of coefficients that must be determined in order to reconstruct the entire piezoelectric tensor at the local scale. Substituting the tensor for \( C_6 \) symmetry into eqn (1) allows for the determination of the real piezoelectric coefficients via a combination of VPFM and LPFM measurements on the three sections of tendon. Solving eqn (2) yields the piezoelectric tensor for collagen in laboratory coordinates.\(^{18}\)

In PFM, we measure:

\[
d_{33} = (d_{31}^0 + d_{15}^0)sin^2\theta cos\theta + d_{33}^0 cos^3\theta \quad (4)
\]

\[
d_{34} = sin\theta [ -2(d_{33}^0 - d_{31}^0 - d_{15}^0) cos^2\theta cos\psi + d_{14}^0 cos\theta cos\psi + d_{15}^0 cos\psi ] \quad (5)
\]

\[
d_{35} = sin\theta [ -2(d_{33}^0 - d_{31}^0 - d_{15}^0) cos^2\theta cos\psi + d_{14}^0 cos\theta sin\psi + d_{15}^0 sin\psi ] \quad (6)
\]

where \( \theta \) is the angle between the long axis of the tendon and the direction of applied electric field, and \( \psi \) is the angle between the long axis of the cantilever and tendon axis.

For the 0° tendon section, the applied electric field is in the same direction as the long axis of the tendon. This allows for the direct measurement of the \( d_{33}^0 \) piezoelectric coefficient (\( \theta = 0^\circ \)). Additionally, when the tendon axis is perpendicular to the applied field and to the long axis of the cantilever (90° section), the \( d_{15}^0 \) piezoelectric coefficient can be directly measured (\( \theta = \psi = 90^\circ \)). To determine the remaining piezoelectric coefficients, VPFM and LPFM were measured from a 45° section of tendon. For \( \theta = 45^\circ \), equation (4) becomes:
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\[ d_{33} = \frac{1}{2\sqrt{2}}(d_{31}^0 + d_{15}^0) + \frac{1}{2\sqrt{2}}d_{33}^0 \]  \hspace{1cm} (7)

Since both \( d_{33}^0 \) and \( d_{15}^0 \) were directly measured using PFM at 0° and 90° sections, it is possible to deduce the \( d_{31}^0 \) piezoelectric coefficient from VPFM of the 45° section. Similarly, if a shear signal is detected from the 45° section, eqn (6) becomes:

\[ d_{35} = \frac{1}{2}d_{14}^0 + \frac{\sqrt{2}}{2}d_{15}^0 \]  \hspace{1cm} (8)

Once the \( d_{15}^0 \) coefficient (which is directly measured) is been determined, it is then possible to deduce the coefficient \( d_{14}^0 \) and thus reconstruct the entire piezoelectric tensor of collagen assuming it has \( C_6 \) symmetry as reported. Standard error propagation techniques were used throughout. 31

7.3.2 Probing real piezoelectric coefficients of collagen using PFM

In the case of the 0° section, where the tendon axis is aligned orthogonal to the cantilever, the \( d_{33}^0 \) coefficient is directly probed using VPFM as \( d_{33} = d_{33}^0 \) for \( \theta = 0° \), as described above. A 3D AFM height image from the 0° section with a mixed VPFM piezoresponse image overlaid, which contains both amplitude and phase information (\( d_{33} \) coefficient map), is shown in Fig 7.3 (a). Regular circular features are visible throughout the image from the height data, each of which may correspond to individual fibril cross sectioned ends. Bright (yellow) and dark (purple) regions represent domains (i.e., regions of uniform phase response) of opposite polarization. The domains have a circular pattern with a diameter which matches the round features observed in the AFM height data within experimental error (domain diameter: 159 ± 26 nm; height diameter: 184 ± 39 nm; \( n = 15 \)). Characteristic domain sizes were calculated via correlation analysis, whereas the
diameters of the ‘round’ features were measured via line profiles of the height data. These values are in good agreement with the average diameter of tendon in 4 week old rat (~170 nm), as measured by EM. This signifies that the typical piezoelectric domain in the cross section of tendon corresponds to an individual cross sectioned fibril end. A gap in the surface is evident on the left of Fig. 7.3 (a) where no domains are visible, likely signifying a boundary between fascicles.

For the orientation where the substrate-bound longitudinal tendon axis is orthogonal to the longitudinal axis of the cantilever ($\theta = \psi = 90^\circ$), the coefficient $d_{15}^0$ can be directly determined using LPFM ($d_{15}^0 = d_{35}$). A 3D AFM height image of this 90° section with the mixed $d_{15}$ coefficient image overlaid is shown in Fig 7.3 (b). Since the measured shear piezoresponse is now sensitive to $\psi$ ($d_{35} = d_{15}^0 \cos \psi$), for all point measurements and images, any deviation from a 90° angle between the cantilever and fibril axis was corrected for using this equation and measuring the angle offset via Igor Pro (Wavemetrics). From the height data, parallel collagen fibrils can be seen which exhibit the well-known 67 nm D-periodicity associated with type I collagen, which was measured to be 66 ± 1 nm by subtracting the background from a representative line profile and fitting to a sine function. A boundary between two fascicles is visible in the image parallel to the fibril axis. The LPFM overlay confirms the expected shear piezoelectricity along the collagen fibril axis. An antiparallel polar orientation of fibrils is observed down to the fibrillar level, similar to that seen previously for rat tendon, fascia, and eye tissues.

Interpretation of the 45° section is more complex as the measured signal consists of a combination of piezoelectric coefficients. A 3D height image of the 45° section with a
The length of the piezoelectric domains here are $615.7 \pm 24.2$ nm along the fibril axis (as measured by correlation analysis and exponential fit), which is around double the expected value for oriented collagen with a diameter of $\sim$170 nm section at 45° ($\sim$250 nm). It is possible these domains consist of both single and double collagen fibrils. However, it is important to note that the nanoscale values quoted here are point measurements, described below, which give a local value and do not scale with the domain size. These nanoscale measurements are limited by the size of the tip radius only, which is significantly smaller than the size of typical domains. This will result in the determination of a nanoscale tensor which is unaffected by domains of opposite polarity and the subsequent cancelling effect.
Figure 7.3 PFM measured surfaces of 0°, 45°, and 90° sections of rat tail tendon. (a) Mixed PFM image ($d_{33}^0$ coefficient map) of 0° section, comprising fibril cross sections. (b) Mixed PFM image ($d_{15}^0$ coefficient map) of 90° section, comprising fibrils parallel to the surface. Note the angular shift between the length of fibril and sample axis (labelled 144
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y) which is corrected for. (c) Mixed PFM image ($d_{35}^0$ piezoresponse map) of 45° section, comprising fibrils at 45° angle to tendon axis. Each section includes expected shape of fibril (or ‘domain’) based on [26].

By combining measurements of VPFM ($d_{33}^0$) and LPFM ($d_{15}^0$) on the 45° section, it is possible to deduce both $d_{31}^0$ and $d_{14}^0$ coefficients. The measured $d_{33}$ signal for the 45° section becomes a combination of coefficients:

$$d_{33} = \frac{1}{2\sqrt{2}}(d_{31}^0 + d_{15}^0) + \frac{1}{2\sqrt{2}}g_{33}^0$$

allowing for the deduction of the $d_{31}^0$ coefficient when $d_{33}^0$ and $d_{15}^0$ are known and directly measured:

$$d_{31}^0 = 2\sqrt{2}\left[d_{33} - \frac{1}{2\sqrt{2}}(d_{15}^0 + d_{33}^0)\right]$$

Similarly, the measured $d_{35}$ signal for this section becomes:

$$d_{35} = \frac{1}{2}d_{14}^0 + \frac{\sqrt{2}}{2}d_{15}^0$$

allowing for the deduction of the $d_{14}^0$ coefficient:

$$d_{14}^0 = \sqrt{2}\left[-d_{15}^0 + \sqrt{2}d_{35}\right]$$

and thus reconstructing the collagen piezoelectric tensor at the fibrillar scale.

7.3.3 Domain size measured via correlation analysis

Simple graphical representations of the expected domain shapes for each section are shown in Fig. 7.4 (a). A 1D autocorrelation function was obtained using the mixed PFM images for each section and the characteristic domain size was calculated in the x and y direction, respectively. It was determined that the domain size in the x direction (fibril
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diameter) for the 0°, 45°, and 90° sections was 160.8 ± 2.4 nm, 198.3 ± 6.2 nm, and 162.7 ± 4.4 nm, respectively. These values are in good agreement considering the assumption that each domain is composed of a single collagen fibril. The domain size in the y direction (fibril length) for the 0°, 45°, and 90° sections was 158.1 ± 2.8 nm, 615.7 ± 24.2 nm, and 5.37 ± 0.03 µm, illustrating the elongation of the domain sizes along the fibril length axis with increasing sectioning angle. The domain sizes for each section are illustrated in Fig. 7.4 (b).

Figure 7.4 Characteristic domain sizes determined via auto-correlation functions of the mixed PFM images displayed in Fig. 7.3 (a) - (c). (a) Simple graphical display of expected domain shapes for 0°, 45°, and 90° sections based on literature values. (b) Characteristic domain sizes plotted for each section along the x and y direction (○ and + respectively).
Piezoelectric coefficients were quantified via local point measurements. The conductive tip was placed in contact with the surface at several \( n = 20 \) locations per sample. Here, the reported values of the collagen fibrillar piezoelectric tensor are the average and standard deviation. The uncertainty inherent in an individual measurement and the uncertainty in the invOLS calculation (obtained by averaging multiple force curves) were also propagated to include in the reported standard deviations. Fig. 7.5 (a) is a typical VPFM response as a function of applied ac voltage obtained for the 0° section. As described by eqn (4) for this case, the slope of this graph yields the \( d_{33}^0 \) piezoelectric coefficient \( (0.9 \pm 0.3 \text{ pm/V}) \). Fig. 7.5 (b) is a representative LPFM response versus applied ac voltage acquired on the 90° section. Eqn (6) as described above simplifies to \( d_{35} = d_{15}^0 \). Thus, the slope of Fig. 7.4 (b) is a direct measurement of the \( d_{15}^0 \) piezoelectric coefficient \( (4.2 \pm 0.7 \text{ pm/V}) \). This result is in agreement with several previous publications.\textsuperscript{17,19}
Figure 7.5 Point measurement graphs displaying piezoelectric responses in the three sections. (a) VPFM response (○) measured on 0° section as a function of applied voltage. \( d_{33}^0 = d_{33} \) (b) LPFM response (□) measured on 90° section as a function of applied voltage. \( d_{35} = d_{15}^0 \). (c) LPFM response (◊) and VPFM response (Δ) measured on 45° section as a function of applied voltage, allowing for the deduction of remaining piezoelectric coefficients.
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The measured $d_{35}$ and $d_{33}$ piezoresponse as a function of applied ac voltage are shown in Fig 7.5 (c). Using eqns (10) and (12) which are solved for the 45° section case for both VPFM and LPFM voltage sweeps, respectively, it was possible to deduce both the $d_{31}^0$ and $d_{14}^0$ coefficients (8.9 ± 0.6 pm/V and -1.2 ± 0.5 pm/V, respectively), thus successfully reconstructing the entire piezoelectric tensor for the case of collagen at the fibrillar level:

$$d_{ij}^0 = \begin{pmatrix} 0 & 0 & 0 & 1.2 \pm 0.5 & 4.2 \pm 0.7 & 0 \\ 0 & 0 & 0 & 4.2 \pm 0.7 & -1.2 \pm 0.5 & 0 \\ 8.9 \pm 0.6 & 8.9 \pm 0.6 & 0.9 \pm 0.3 & 0 & 0 & 0 \end{pmatrix}$$  \hspace{1cm} (13)

These results show an apparent discrepancy between electromechanical measurements recorded at the nanoscale and the macroscale. The tensor previously calculated at the macroscale for tendon $^7$ ($d_{33}^0 = 0.0866$ pm/V; $d_{31}^0 = 0.066$ pm/V; $d_{14}^0 = -2.66$ pm/V; $d_{15}^0 = 1.4$ pm/V) appears to greatly underestimate most coefficients (notably $d_{33}^0$ and $d_{31}^0$) when compared to the nanoscale coefficients we report ($d_{33}^0 = 0.9$ pm/V; $d_{31}^0 = 8.9$ pm/V; $d_{14}^0 = -1.2$ pm/V; $d_{15}^0 = 4.2$ pm/V) which are almost all higher than their macroscopic counterpart.

The average piezoresponse of $d_{33}^0$ images ($n = 10$, typically $8 \times 8 \mu$m) was calculated to be $-0.13 \pm 0.19$ pm/V, highlighting the problem that macroscopic measurements underestimate coefficients due to the piezoresponse from oppositely oriented fibrils cancelling out. This is further supported by histogram analysis of the images, which yields an average difference between the amount of domains pointing up versus down (comprising opposite N to C polarities of the fibrils) of 17% (phase difference). This leads to an effective signal being measured (in the macroscopic and even microscopic case) which underestimates, or even negates coefficients. Thus, despite measured local
response on the order of 1 pm/V, the measured averaged response at the microscale (as measured via averaging multiple images) is lower, in line with macroscopic measurements, suggesting a similar mechanism.

The LPFM values presented in eqn (13) are based on a geometric lateral calibration. Other calibration methods are based on torsion curves via increasing scan sizes to represent increasing tip torsion,\textsuperscript{33} and comparison with a standard sample such as γ-cut LN.\textsuperscript{28} These methods are discussed in more detail in section 2.2.3.2. Applying the former method reduces shear coefficients by one order of magnitude while the latter leads to an increase of one order of magnitude. Another calibration method involving a direct measurement of lateral signal as a function of surface angle tilt, also described in section 2.2.3.2 was used on these results. It was discovered that when using this calibration method, the shear piezoelectric coefficients are reduced. The $d_{15}^0$ coefficient is reduced to $\sim 0.2$ pm/V, which leads to a reduction in the $d_{31}^0$ and $d_{14}^0$ coefficients - as they become $\sim 0.8$ and $0.1$ pm/V respectively. As discussed in chapter 6, there exists a huge disparity in the measured shear coefficients across the whole spectrum of LPFM users. More work needs to be undertaken by the community in order to standardize LPFM calibrations and to agree under which circumstances calibration standards should be used. Notably, the discrepancy between lateral calibration techniques only affects the shear coefficients. Similarly, the vertical response can be calibrated using $x$-cut quartz ($d_{11}^0 = 2.2$ pm/V),\textsuperscript{34} to yield a $d_{33}^0$ coefficient of collagen of $1.1 \pm 0.2$ pm/V, which is within the error of the force curve calibration method used above.

The piezoelectric tensor for collagen at the fibrillar scale has been determined for the first time. The results reported here have implications for piezoelectric measurements.
performed at the macroscopic scale in the case where the sample contains domains of oppositely oriented polarizations. We have seen that in these cases, piezoelectric coefficient values can be greatly underestimated or even negated. The refined piezoelectric tensor of collagen at the fibrillar level will be important for future studies involving associated biofunctionalities of piezoelectricity in collagenous materials as the cellular responses to stress and electrical stimuli occur, and are sensed, at the local scale. Importantly, the $d_{33}^0$ of an individual collagen fibril is $\sim 10$ times greater than previously reported for tendon macroscopically, suggesting applications based on the longitudinal piezoelectric response are viable.

7.4 References


Chapter 8

Summary and future work

8.1 Summary

In this thesis, piezoresponse force microscopy was utilized to study collagen fibrils and collagenous tissues in order to better understand their electromechanical properties. A main objective was to understand better the mechanism of piezoelectricity in collagen in order to address possible biofunctional significances of piezoelectricity in any collagenous connective tissue. Determining the polar architecture of natural tissues was another main objective, since information of which is lacking in the literature due to the destructive nature of staining techniques which are needed to visualize polarity in tissues. PFM eliminates the need for staining so the non-destructive imaging of the polar properties of natural tissues was achieved.

Advancing our knowledge of the electromechanical properties of collagen allow for a new class of biomedical devices and implants to be designed which can implement this naturally occurring functional property. PFM was demonstrated on a variety of
collagenous samples, ranging from a collagen fibril to both natural and assembled collagenous tissues and membranes. Piezoelectric domain imaging, the quantification of piezoelectric coefficients, effect of pH and structure on electromechanical properties was all investigated. Of all the studies explored, the following are the main conclusions which deserve particular attention:

1) Shear piezoelectricity was confirmed in all collagenous samples studied – by combining vertical and lateral PFM, the polar architecture in tissues can be visualized. Interestingly, in all tissues studied – rat tail tendon, porcine cornea, sclera and iris – no uniform polarity was observed, which was previously unknown in natural tissues. Rat tendon showed evidence of anti-parallel polar ordering down to the fibrillar level, which has been confirmed to persist partially through the thickness of the tissue. The complex polar architecture revealed in all tissues studied may have huge implications in vivo regarding cell signaling and resultant tissue development, as the polar organization of collagen can provide a cue for cell response.

2) The role of pH and structure were discovered to have a significant effect on the electromechanical properties of collagen. Changing the pH from acidic (pH = 2.5) to neutral (pH = 7) led to a 1.35 fold increase of the lateral piezoresponse of a collagen nanofibrils membrane in addition to a structural change, namely the formation of a crimp structure perpendicular to the alignment of collagen nanofibrils. These membranes were shown to influence cell behavior previously, so the change in the functional properties of the membrane may also influence cells. Shear piezoelectricity was also confirmed in a non D-periodic collagen
membrane, contradicting the literature which attributes piezoelectricity to the hexagonal packing of collagen molecules (may only be present in D-periodic fibrils).

3) Shear piezoelectricity was demonstrated to persist in an assembled collagen hydrogel at the fibrillar level – and the results were directly compared to the control, rat tail tendon. The dried hydrogel successfully replicated the characteristic D-period associated with type I collagen fibrils. A higher piezoelectric signal was observed in the dried hydrogel which may be related to the lack of cross-links present in the hydrogel. These observations are important for the tissue engineering community as assembled/engineered tissues should replicate both structural and functional properties of the intended replacement. Piezoelectricity in engineered tissues is not addressed, yet the presence or absence of this property could influence cellular behavior. In addition, in vitro cell culture experiments using collagen substrates should consider the effect of piezoelectric collagen in particular for stress studies.

4) Collagen type II, abundant in cartilage, was shown via PFM to be a shear piezoelectric, exhibiting an angle dependence of the piezoelectric signal with cantilever-fibril angle. The shear piezoelectric coefficient of type II collagen was determined to be ~ 30% lower than that measured for collagen type I with the same tip/conditions. The piezoelectric properties in collagen type II were previously unknown, so further studies should aim to discover if this property has a functionality in cartilage, since charges have been shown to play a role in the
mechanical properties of cartilage, in addition to stimulating chondrocyte proliferation.

5) The piezoelectric tensor of collagen was determined at the nanoscale via sectioning rat tail tendon at angles of $0^\circ$, $45^\circ$ and $90^\circ$ relative to the major axis of the tendon and measuring the piezoelectric properties via PFM. All piezoelectric coefficients were found to be larger than those previously reported at the macroscopic scale. This new local tensor may be used for future studies which concern the biofunctional implications of piezoelectrically-induced charges in collagenous tissues.

8.2 Future work

While the scope of this thesis has been to further our knowledge on the manifestation of piezoelectricity in collagen, there exists a pressing need to continue to explore the possible biofunctionalities of piezoelectricity in collagen, and the role it plays in vivo. Several experiments can be tailored to investigate these problems via scanning probe microscopy and should be explored in the future.

8.2.1 Determining the role of hydration on piezoelectricity in collagen

One such experiment is the determination of the role of hydration on piezoelectricity in collagen. This is important since there are several macroscopic reports claiming collagen loses its piezoelectric nature when fully saturated, yet more recent nanoscale measurements on saturated bone revealed a longitudinal piezoresponse. Piezoelectricity in collagen has been shown to generate sufficient charges to produce biochemical effects
in dry conditions\textsuperscript{3} so it is of high importance to determine conclusively if collagen remains piezoelectric in a hydrated condition, which is relevant for physiological conditions.

Preliminary results using both single frequency PFM and a multi-frequency technique called band excitation PFM (BE-PFM)\textsuperscript{4} were obtained on collagen membranes as a function of relative humidity (RH). An illustration of the experimental setup is shown in Fig. 8.1 below.

![Figure 8.1 Illustration of experimental setup of BE PFM measurements of collagen as a function of humidity.](image)

Since many factors (change in tip-surface forces, mechanical properties etc.) can dramatically influence the measured electromechanical signal in single frequency mode, BE PFM was also implemented. BE allows for the entire resonant frequency response of the cantilever and the transfer function to be obtained, including tip-surface interaction forces (related to frequency shift) and dissipative forces (related to quality-factor). Preliminary single frequency results are shown in Fig. 8.2.
Figure 8.2 AFM topography and LPFM amplitude images of collagen membrane shown at both ambient (a) and 70% RH (b) conditions. (c) Graph displaying the normalized LPFM amplitude of the images as a function of RH.

Fig. 8.2 shows single frequency LPFM images obtained as a function of increasing RH. Collagen fibrils organize themselves into piezoelectric domains, as seen in Figure 8.2 (a), exhibiting the expected shear piezoresponse. Shear piezoelectricity is seen to persist up to 70% RH as seen from Fig. 8.2 (b), which is higher than the moisture content of collagen in bone (cited at 12% - corresponding to ~40-50% RH). For a more complete overview of the interactions happening as the RH is increased, BE PFM was implemented.
Figure 8.3 AFM topography and BE PFM amplitude images of collagen membrane shown at both (a) ambient and (b) 90% RH conditions. (c) Averaged BE amplitude and frequency of each image as a function of ramping humidity.

The lateral BE amplitude images at ambient and 90% RH shown in Fig. 8.2 (a) and (b) confirm that collagen is still piezoelectric at a humidity as high as 90%. The topography images at ambient and 90% RH, Fig. 8.2 (a) and (b) respectively, show that the collagen fibrils have swollen in the moisture-rich environment. Graphs mapping the change in amplitude and resonant frequency as a function of ramping humidity are shown in Fig. 8.2 (c). No statistical change is seen in either the piezoelectric amplitude or resonant frequency (highlighting stable tip-surface interactions). Adhesion forces were
also studied in order to investigate their effect on the PFM signal. Force curves in voltage on and off states were taken to measure any change in the adhesion between the tip and surface meniscus.

![Adhesion as a function of humidity](image)

Figure 8.4 Measured adhesion as a function of humidity in voltage on and off states.

The adhesion was measured via force curve, by quantifying the ‘pull-off’ force required to overcome the capillary force present from the meniscus between the tip and surface. Fig. 8.4 reveals there is no statistically significant difference between voltage on and off states, highlighting that adhesion forces do not affect PFM measurements.

While these results are preliminary, they are encouraging in that there is solid evidence here that collagen remains piezoelectric up to relative humidities of 90% RH. More experiments calculating the mechanical properties in addition to electromechanical properties as a function of humidity could be beneficial in order to determine if the elastic properties of collagen can influence their piezoelectric properties (assuming the mechanical properties of collagen change with increasing moisture content). These
measurements would lead to a final goal of probing collagens electromechanical properties in a liquid environment.

8.2.2 Directly measure the piezoelectrically-induced redistribution of charge in collagen

As discussed in several chapters in this thesis, the most likely mechanism by which piezoelectricity in collagen will directly trigger a cellular response, or indirectly result in protein adsorption, is by a piezoelectrically-induced redistribution of charges. By designing an experiment using an AFM-compatible sample stretching stage; it would be possible to measure the effects of stress and resultant charge. Ideally this could also be implemented for frequency studies, dynamically stretching the collagen sample in the range of frequencies relevant for human locomotion. Using standard charge measuring AFM techniques such as electrostatic force microscopy (EFM) and Kelvin probe force microscopy (KPFM), the strain induced charge could be measured locally.

8.2.3 Role of piezoelectricity in mineralization

Mineralization within collagen is critical for normal mechanical properties of tissues such as bone and dentin.\(^6\) Huge research efforts are geared towards the understanding of how apatite forms \textit{in vivo}. Currently, the mineralization process can be characterized using TEM – a technique requiring complex sample preparation and must operate under a vacuum. In this regard, applying AFM to investigate mineralization is a promising route. As discussed throughout this thesis, it is as yet unknown how piezoelectricity affects the mineralization of tissues; therefore in the future it would be excellent to use AFM in combination with PFM and an elastic-sensitive technique such as atomic force acoustic microscopy (AFAM) to investigate the electromechanical and mechanical properties of
remineralized dentin. It could be possible with this combination to detect mineral in collagen without the use of TEM. In addition, using an AFM-compatible sample stretching stage as discussed in section 8.2.2, in combination with strained collagen in calcium phosphate solutions, mineralization of strained fibrils can be explored.

8.3 References